The Cytoplasmic Inclusions of the Germ-Cells.

PART II. HELIX ASPERSA.

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With Plates 29–34 and 5 Text-figures.

Table of Contents.

PAGE
1. Introductory 556
2. Previous Work 556
3. Technique 562
4. The Germinal Epithelium 564
5. Cell Generations in the Snail . . . 571
6. Manner of Metamorphosis of an Indifferent Germinal Epithelial Cell into a Primary Spermatogonium . 578
7. Manner of Metamorphosis of an Indifferent Germinal Epithelial Cell into an Oogonium . . . 587
8. The Fate of the Mitochondria and Nebenkern in the Egg 589
9. Manner of Metamorphosis of an Indifferent Germinal Epithelial Cell into a Nurse or Yolk Cell . . 590
10. Description of the Scheme of the Ovotestis Lumen drawn in Pl. 29, fig. 1 591
11. Discussion under the heads of:
    (*a*) Nebenkern 594
    (*b*) Mitochondria 598
    (*c*) The Determination of Oogonium Spermatogonium or Nurse-Cell 599
    (*d*) The Different Male Generations in the Ovotestis . 601
12. Summary 605

INTRODUCTORY.

THE first part of the present series of papers (**1**) dealt with the cytoplasmic bodies in the gametogenesis of the Lepidoptera. In this paper I am publishing the results of a study of the cytoplasm in the hermaphrodite, Helix aspersa. Not only is the gametogenesis treated, but a careful study has been made of the problems surrounding the appearance of eggs, spermatozoa, nurse-cells and follicle cells from the same germinal epithelium. Special care has been taken in order to get the very best preparations of all stages, and extensive experiments were undertaken to improve the current technical fixing methods. This study proved more formidable than at first seemed probable, and some of the minor problems had to be imperfectly treated in order to keep the paper within reasonable bounds. Where such minor problems appear I have indicated in the text, and the snail still offers many attractive lines of research on germ-cells. I have to thank Dr. Goodrich for his kind and constant interest and useful criticism. This work was done in the Department of Physiology, and my warmest thanks are due to Prof. Sherrington.

PREVIOUS WORK.

Apart from the ordinary study of the formation of the gametes, the snail has provided the material for a host of studies on hermaphroditism. The ovotestis, ever since its discovery, has fascinated many observers, and the large number of papers which have appeared on the hermaphrodite gland of the Pulmonates is justified when one considers what a remarkable phenomenon is the appearance of several distinct categories of cells—sperms, eggs, and nurse-cells—from a single indifferent pavement epithelium.

Those who would study the history of our knowledge of the Helicid gametogenesis may consult Ancel's excellent memoir, wherein are mentioned the main contributions made by the large number of observers on this subject. In this paper I only intend to review Ancel and the subsequent writers. (Bolls Lee is mentioned in the text.)

Since I have not examined embryonic stages I give Ancel's conclusions somewhat fully.

Ancel (2) describes the genital rudiment as appearing in the embryo several days before hatching. At first the mass of cells, quite solid, slowly elongates, and ultimately fuses with the hermaphrodite canal. Soon afterwards a lumen appears in the solid mass of cells, which now appear like an irregular cubical epithelium lining an opening, and these cells become covered on their outside by a layer of the mesoderm cells in which they lie. At different points inside the lumen the genital cells begin to divide mitotically, and in the region of these buds secondary, tertiary, etc., culs-de-sac make their appearance. In this way the hermaphrodite gland is built up from a solid rudiment.

The cells which line this gland consist of a single layer. Here and there some of the indifferent cells augment in volume, the chromatin lumps of the nucleus fuse with one another, and give rise to more or less round structures. Ancel calls these cells "cellules progerminatives indifferentes."

Part of the chromatin of these cells loses its affinity for nuclear stains, and the chromatin lumps become more numerous and become rounded. They are now united two by two by nuclein filaments.

All the chromatin of the indifferent progerminative cell condenses into several round bodies. The whole cell grows in size, the nucleus more than the cytoplasm. This cell is called by Ancel the male progerminative cell. The latter begins to divide indirectly, the chromosome number being forty-eight. The products of these divisions drop into the lumen of the ovotestis; they are large and pedicellate (pediculé), and have the nucleus in the larger part of the cell. These primary spermatogonia go on dividing and give rise to much smaller bodies—the secondary spermatogonia. The products of this activity soon fill the lumen of the ovotestis, and in the germinal epithelium changes take place. The cells become arranged in two layers, and the outer layer in contact with the contents of the lumen becomes filled with grains stained with osmic acid, and the nuclei of this outer layer have their chromatin condensed into the form of crowded blocks. These cells are the nurse-cells.

When the nurse-cells are formed certain elements in the inner layer beneath the nurse-cells augment in size. They undergo the progerminative indifferent stage, but then become altered in a different manner. The chromatin lumps of the indifferent cell break up and become oriented to the periphery. The centre of the nucleus then looks clear, while all around its periphery are collected little nuclein nucleoli (sic), united one to another by chromatin filaments. In this cell it is the cytoplasm which has grown most in size. Such is the history of the oocyte. This element does not undergo division before the maturation period. Ancel does not find in Helix the stages of primordial ovum or oogonium described in other oogeneses.

According to Ancel the presence of nurse-cells is a sine quâ non for the formation of an oocyte, and is explanatory of its development.

Concerning the growth period of the oocyte Ancel writes in the following sense: The chromatin of the young oocyte condenses into the form of little nucleoli, which become disposed around the periphery of the nucleus. A network becomes established at the expense of the nuclein nucleoli, and a new network is formed afterwards from the chromatin. In an involved statement Ancel describes the appearance of parachromatic nucleoli, distinguished from the nuclein nucleoli by double staining in hæmalum and safranin.

In the oocyte at an early stage one can find filaments in the cytoplasm. These filaments are originally dispersed through the cytoplasm, but later condense towards a peripheral zone, which diminishes more and more and disappears.

During this time the whole cytoplasm takes on an alveolar appearance except at one place, where special bodies will soon put in an appearance. Hæmatoxylin or safranin preparations show that in this region one soon finds filaments of a variable size. These rods or filaments are known as "corps intracytoplasmiques." The latter seem to be either solid rods or formed of moniliform structures placed in a series.

Ancel thinks that the young spermatocytes do not contain a Nebenkern. Their cytoplasm is filled with short, colourable filaments. The Nebenkern, which appears at the expense of the cytoplasmic filaments, augments in size as these filaments disappear. At the beginning of mitosis the Nebenkern fragments and disappears. Ancel denies that the Nebenkern is related either to the nucleus, the spindle, or the "sphère attractive," and gives it as his opinion that this curious body "ne représente, à notre avis qu'une phase de l'evolution des formations intracytoplasmiques."

Elsewhere Ancel states: "Le Nebenkern ne jouerait donc aucun rôle dans les cellules; il ne serait que le produit de transformation des filaments cytoplasmiques differenciés auxquels serait dévolue une fonction spéciale."

R. Demoll (**3**) describes the fact that these are prophases of the heterotypic division in the female, which was overlooked by Ancel. He describes the Nebenkern somewhat better than Ancel or previous authors and considers that this body determines the sex of the differentiating cell.

Writing of the "Bukettstadium in beiden Keimzellen," Demoll gives the following: "Mit dem Ausstofen des Nebenkernes wird sowohl die Wachstumsgeschwindigkeit als auch die Genese der chromatischen Substanz für die beiden Arten von Keimzellen eine spezifische. Oder: Der Nebenkern bedingett erst die geschlecht liche Differenzierung der bis zum Bukettstadium indifferenten Keimzellen." In the discussion it will be shown that for a number of good reasons Demoll's sex determination hypothesis cannot be accepted (page 40).

Iw. Buresch (**4**) has also attacked the problem, but his paper contains little of interest from the point of view of the means whereby sex is determined. His work is not a great advance on that of Ancel, written many years before, while he has failed to study any cytoplasmic inclusions. Buresch thinks the germinal epithelium is a syncytium, but as he uses Zenker for fixing this is almost certainly due to the brutality of fixation. Buresch gives a fairly good account of the generations of egg-cells, nurse-cells, etc., and shows how the presence of a large egg affects the surrounding cells.

Concerning forms other than Helix we have two late papers by Terni and by Schitz. The latter (**5**) on Columbella finds the Nebenkern rods in the spermatocyte at the beginning of growth, and in later stages they form a sort of palisade around the archoplasm (idiozome). In the prophases of the maturation mitoses these rods disappear. The mitochondria, before seed-like, become elongate as in the snail during mitosis. The Nebenkern in spermatogenesis becomes passed into the tail region where it "ultimately degenerates," but the author does not pay much attention to late stages.

Schitz somewhat obscurely describes the formation of an acrosome from a "grain siderophile," derived from the idiozome (archoplasm), and to support his statement quotes Champy's demonstration of the fact that in Amphibia the acrosome develops at the expense of one of the central corpuscles. I find it difficult to accept Schitz's claim, and his figures are not conclusive enough. The mitochondria form a sort of double sac-like structure, "envelloppant le filament axile."

Finally, Schitz speaks of the tail as "constituée par le filament axile, entouré de sa gaine mitochondriale." It will be seen that, excepting the formation of the acrosome, the fate of the Nebenkern and its behaviour throughout, according to Schitz, corresponds to what happens in Helix.

Tullio Terni (**6**) in Geotriton carefully describes Nebenkern and mitochondria. In divisions the Nebenkern fragments or completely loses its original character. Terni having described the fragmentation of the batonettes proceeds to discuss the question as to the origin of the spindle. Nebenkern and idiozome seem synonymous to him, for I find "Il Nebenkern dei Gasteropodi polmonati (Idiozoma)." He says:

"Sostenendo l'origine endoidiozomica (Terni calls the batonettes 'formazioni periidiozomiche,' and elsewhere 'dittosomi') del fuso centrale, vengo ad ammettere che l'idiozoma abbia un importante ufficio nella formazione del fuso stesso: forse di significato assai più profondo che non quello di una semplice azione protettiva. Non ho però dati sufficienti per definire la portata reale della partecipazione materiale dell'idiozoma alla formazione del fuso centrale; credo tuttavia che la sostanza idiozomica non si esaurisca tutta nella produzione del giovane fuso centrale." In a footnote Terni informs the reader that in his work he could not demonstrate the existence of "due mezzi fusi periferici nella profase dell prima divisione di maturazione."

Terni definitely describes the formation of an acrosome from a part of the archoplasm. In this he is in agreement with what Schitz found in Columbella. As I have already said I cannot find such a process in the snail, but I am quite open to conviction that the idiozomatic body contains the future perforatorium, though before coming to a conclusion I should like to have further evidence either way. I am quite certain that in the snail the archoplasm (and its Nebenkern rods) does not approach the head of the nucleus in the way described by Schitz. In Terni's case the nucleus seems to revolve somewhat after the acroblast has become stuck on one side. The acroblast is thus brought to the head end of the cell. Terni's case seems very clear.

Fauré-Fremiet (7) in his comprehensive article, "Etude sur les mitochondries des Protozoaires et des Cellules Sexuelles," gives figures and describes the mitochondria of Helix pomatia and Arion rufus.

He fails to discover the micromitochondria, overlooks the acrosome, and gives unproportionate drawings of Nebenkern rods and chondriosomes. He definitely draws the batonettes at the poles of the maturation spindle.

Though the minute cytology of the germ-cells of Helix has been unsuccessfully dealt with by Fauré-Fremiet, the real value of this observer's work lies in his remarks on the Nebenkern in fresh preparations. He says: "Il m'a semblé pourtant qu'il (the Nebenkern) était beaucoup plus facilement

altéré que les mitochondries environnantes par les solvants des graisses; les mitochondries resistent parfaitement à ceux-ci, et j'ai noté qu'un spermatozoide d'Arion traité après dissociation par l'alcool et le chloroforme et coloré par la méthode de Mallory montre les deux cordons mitochondriaux se colorant par la fuchsine acide avec énergie."

Technique.

All the earlier observers hardly without exception found that Flemming's strong formula gave the best results of any fixatives at their disposal.

Murray (8) got good results with Perenyi. Flemming's unmodified formula I consider out of date and worthless for a study of the cytoplasm. Its fault lies in the presence of acetic acid, a reagent which should never be used for work on the plasma. As I explained in my last paper, a modification, which I found best, was to cut out all the acetic acid.

Meves, in his great cytological work, used a Flemming of this sort: 15 c.c. of chromic acid of 1 per cent., containing NaCl of 1 per cent., and 3 to 4 c.c. of osmic of 2 per cent., with three or four drops of acetic acid. Benda used a Flemming with only three to six drops of acetic acid to 15 c.c. chromic and 4 c.c. osmic. I feel sure that it is dangerous to try to temporise between two fixing solutions, as nuclear and cytoplasmic, by simply cutting down the acetic. These observers retain just enough acetic acid to distort the mitochondria, and the small improvement in the appearance of the nuclei and in the penetrative power is quite outweighed by this glaring fault. As I have mentioned before, the discussion as to whether the mitochondria are rods or granules may be largely a question of acetic acid and such-like injurious reagents.

Any fixative in general use will give a passable fixation of the nucleus, but work on the cytoplasm is a very different story. In the cytoplasm we have no bodies bounded by close membranes, but structures which can be, and always are, blasted into fragments by many so-called "admirable fixatives."

In this work on the snail I used the following fixatives:

(1) My modification of Flemming's strong formula, in which no acetic was used.

(2) The same fluid diluted one-sixth.

(3) Champy's fluid.

(4) Flemming's strong solution, in which the acetic acid was re-placed by nitric acid.

All the better-known fixatives were also tried, such as Flemming, Perenyi, corrosive sublimate, Carnoy, etc.

For staining I used the usual stains, such as Ehrlich's hæmatoxylin and eosin, Mayer's acid hæmalum, and iron alum hæmatoxylin with orange G., Bensely's acid fuchsin-methyl green, and alizarin-toluidin blue, etc.

Taking the reddish or orange alizarin or acid fuchsin as a basis, the slides were often counterstained in toluidin blue, crystal violet, methyl green, thionin, etc., and the same was done taking iron hæmatoxylin as the first stain. Some slides were mounted unstained in euparal for studying yolk.

Smears were tried, but failed to give very helpful results except for studying later stages and ripe spermatozoa.

Of the large number of fixing and staining methods used, I found that for the snail the following was the best: The animals are anæsthetised in chloroform vapour, and as soon as possible the last upper whorls of the shells are carefully broken with strong forceps or by a blow with some hard instrument. The shell is dipped in water, and the remaining pieces around the broken area are removed with forceps. The ovotestis is cut out and laid on the table and superfluous digestive gland is cut away, exposing the milk-coloured ovotestis on every side. This piece is cut in half longitudinally, and immediately thrown into a capsule of Flemming without acetic acid, diluted one-sixth with distilled water. Here the fragments are left overnight. They are washed for at least two hours in running water next morning, and then brought up from 50 per cent. alcohol and the other grades to absolute and xylol. They are embedded as usual, cut into 6 μ sections, and stained in iron alum hæmatoxylin by the long method.[1] These sections are carefully differentiated, and then tinged with orange G. or van Giesen. The mitochondria and Nebenkern are intense black and beautifully clear.

Neither Benda's, Bensely's, nor any of the other coloured stains approach such preparations for definition, and for the detail which is shown. As will be explained in another part of this series, iron hæmatoxylin is not always indicated for mitochondrial work, Bensely's acid fuchsin having been found better for some objects.

The Flemming solution, in which the acetic acid is substituted by nitric acid of 3 per cent., gives a very intense stain when used on the mitochondria.

The Germinal Epithelium.

The germinal epithelium in its indifferent condition consists of a row of flattened cells containing compressed nuclei. The epithelium is not a syncytium, as has been stated by some authors. In Pl. 30, fig. 2, is drawn at a very high power three cells of the epithelium in an indifferent state. The nucleus is an oval, flattened structure, and as it is here cut in its narrowest way it looks elongate. The chromatin is arranged in a large number of irregularly triangular lumps, which here and there are intercommunicating. The cytoplasm is not very large in volume, and consists of a wide reticulum, which in some cases can be seen to be condensed into a dark mass near one edge of the nucleus (see Pl. 30, fig. 2, Pl. 31, fig. 20, etc.).

The cells of the germinal epithelium rest upon a fibrous layer, shown by Ancel to be of mesodermal origin (Pl. 30, figs. 2, 3, and 4, *A.L.N.*, etc.). This layer contains nuclei, which vary in size very greatly. In Pl. 30, figs. 3 and 4, are drawn quite typical examples of the germinal epithelium. The germinal epithelial cells (*G.E.*) are seen resting between the yolk cells (*N.C.*) and the layer of Ancel (*A.L.N.*). According to the sort of sex cells in any given region the germinal epithelium is characteristic. Where rapid proliferation is taking place, where yolk cells are abundant, or where a large oocyte is present, the epithelium has a special appearance. In Pl. 30, figs. 3 and 7, and in Pl. 31, figs. 9, 11, and 12, where an oocyte is in growth, the germinal epithelial cells tend to either become atrophied or altogether pushed aside. In Text-figs. 1, 2, 3, and 4 are drawn typical portions of the ovotestis under different conditions. It will be seen that the appearance of an alveolus of the ovotestis may vary greatly. Not only does the wall differ at different stages, but the contents of the lumen are rarely the same in appearance. I believe that the varying different stages in the alveoli can be classified for different seasons of the year, though if the ovotestis is sectioned during hibernation it will be found that all the various sorts of alveoli are present.[1] Then one is led to inquire what causes individual alveoli of the ovotestis to vary so remarkably in appearance as do those drawn in Text-figs. 2 and 3. Ancel has given a description of the metamorphosis of the alveolus in the young snail, which, in view of his Flemming technique, is not very satisfactory. Unfortunately his methods did not allow him to do anything but describe the nuclei, but from his otherwise admirable description we know that the following events take place :

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[1] Iron alum ten to twelve hours, hæmatoxylin twelve to fourteen hours.

Firstly the male progerminative cells appear and drop into the lumen. Then the germinal epithelium becomes arranged in two layers, the inner of which remains indifferent, the outer (next to the male cells) becoming converted into nurse-cells. Thirdly, the inner layer of an indifferent nature sporadically gives rise to oocytes. That this is really what happens I have no doubt, and if one keeps this description of the sequence of events in one's memory, some difficult problems with which one meets in studying the adult ovotestis become less hard to understand.

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[1] But the orders of cells differ in the seasons. This matter is dealt with in a forthcoming paper.

In the ovotestis of the hibernating snail where activity is temporarily suppressed, just as in a summer example where activity is very great, it is found that the diverticula or alveoli in the hermaphrodite gland almost always are better provided with yolk and younger cells at their upper extremity than at the lower part of the finger-like alveolus which joins the mouths of other diverticula. That is to say, the higher ones penetrate into the diverticulum, the younger and less differentiated are the elements. When one cuts a transverse section across the upper part of an alveolus one finds that the lumen is very small and is choked either with full yolk cells projecting from the walls, or closely packed with spermatogonia and young spermatocytes. It is rare to find an oocyte at these places, but it would be a mistake to think that oocytes never occur in these regions. In Text-fig. 1 is drawn the upper region of an alveolus. Just as in the young snails described by Ancel, the first cell elements to appear are almost invariably spermatogonia, but I have found several instances where an oocyte had appeared immediately after the yolk cells had been formed. In Text-fig. 1, i, the germinal epithelium has already become organised into two layers, an inner mass of yolk cells filling the lumen and the lower indifferent germinal cells (*G.E.*). At *X.* a germinal epithelial cell has grown in size, has lost its flattened shape and is about to become a spermatogonium. In Text-fig. 1, ii, this process has become more advanced and the nurse-cells are becoming pressed apart at *X.* by a number

Text-fig. 1.

Fig. i.—Upper part of diverticulum of ovotestis showing yolk cells (N.C.) and germinal epithelium (G.E.). At X. are enlarged nuclei, which are in a progerminative stage. In the middle are some spermatocytes. The vacuolised tissue outside consists of mesoderm, which packs around the upper parts of the diverticula. × 800. Fig. ii.—Another diverticulum cut near its upper end. At X. are some primary male cells which as yet have no definite mitochondria or Nebenkern. They will probably become spermatocytes directly. At Y. is a pale cell beginning to enter a primary spermatogonial stage. Figs. iii and iv are spermatids showing different Nebenkern batonettes (N.K.). M^1. = micromitochondria. M^2. = macromitochondria. N.C. = yolk cell.

TEXT-FIG. 1.

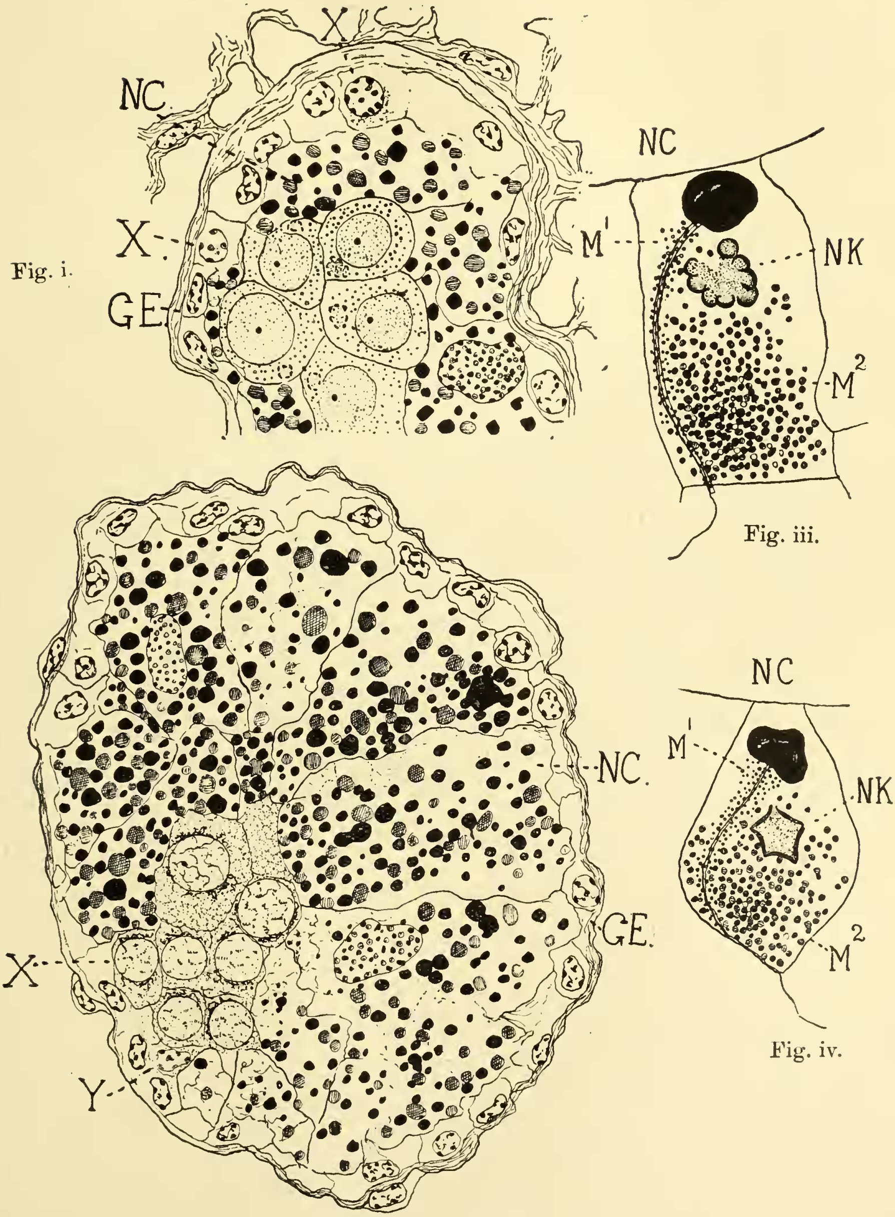

Fig. i.

Fig. iii.

Fig. iv.

Fig. ii.

of spermatogonia. This process takes place from different parts of the alveolar wall and the lumen becomes quite choked with cells. In Text-fig. 2 is drawn a lower part of the alveolus showing that the proliferation of male cells from the walls has stopped temporarily and that the yolk cells are much reduced in size.

The consecutive stages from spermatogonium to spermatocyte are marked with figures. Quite often one finds oocytes developing beneath the yolk cells. In such a case as the group of spermatogonia marked i, in Text-fig. 2, many of the cells may go on to the growth period and ultimately form spermatozoa, but there is hardly any doubt that some remain quiescent, or keep on dividing and give rise to the very small spermatogonia with hardly any cytoplasm which are so characteristic of the lower part of the ovotestis lumen. In Text-fig. 3 another stage is reached. The yolk cells are either relatively few or small, or often not present at all, but the lumen is generally strewn with a mass formed of the flotsam and jetsam of many generations of germ-cells of ripe spermatozoa, of exhausted yolk cells or their nuclei, and of many sizes of spermatogonia; also here and there around the walls may be seen either an oocyte in process of growth, often but not always accompanied by yolk cells, or a bunch of spermatocytes, spermatids, or spermatozoa. If such a stage as the contraction figure be taken, one is struck by the fact that many sizes of cells are undergoing this nuclear change, often with a cytoplasm and nucleus varying remarkably in size. In Text-fig. 2 there is no doubt that all the cells are of

TEXT-FIG. 2.

Fig. i.—Slightly lower region than that drawn in Text-fig. 1, ii. All cells belong to the same generation. Earliest stage at 1 has just finished division. 2–7 are growth stages. 8 = spermatid. The yolk cells (*N.C.*) are beginning to become exhausted. The Nebenkern rods of this generation were semi-lunar in shape. Fig. ii.—Further down the diverticulum, where the yolk cells are more exhausted or absent. The epithelium at the figures 1–5 is beginning to produce another generation of male cells. 5 is about to enter the prophases of mitosis. 7 = spermatocyte, 8 = spermatid. The cell 2 is drawn in Pl. 31, fig. 17.

TEXT-FIG. 2.

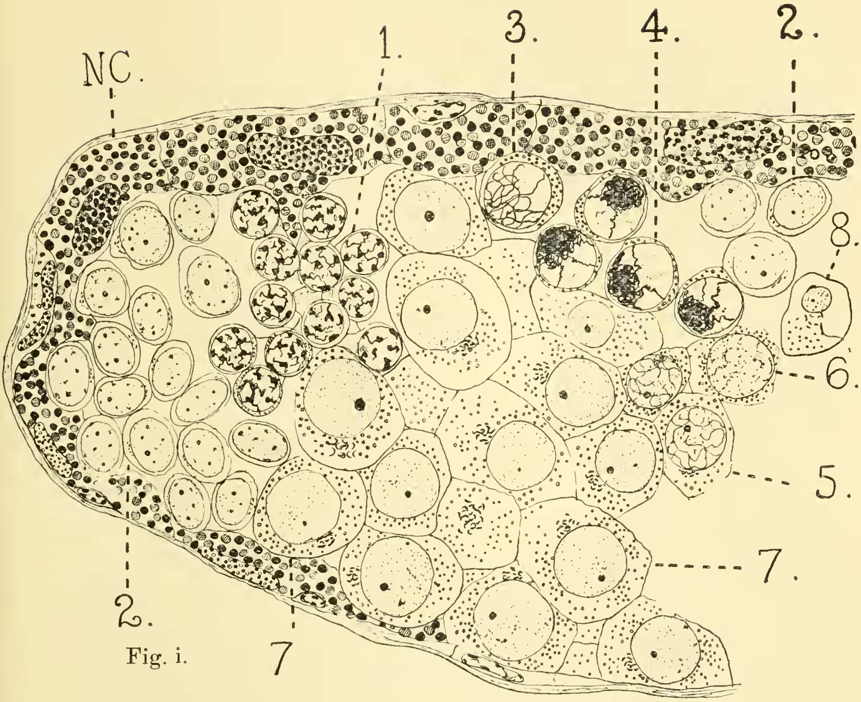

Fig. i.

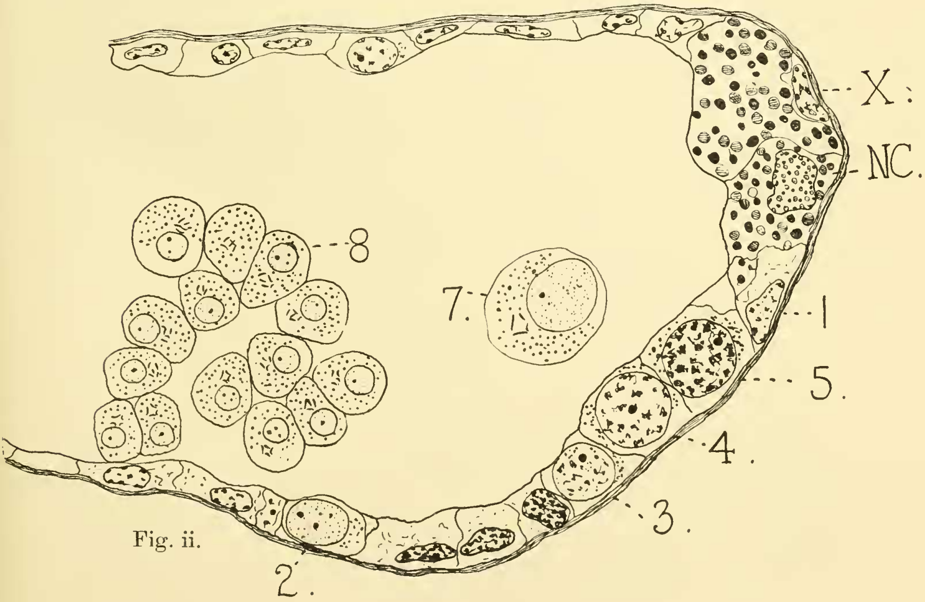

Fig. ii.

the same generation. This is never the case further down the lumen. We then come to one of the most remarkable facts which I am able to point out in this paper. It is that the spermatocytes, etc., in different parts of the lumen are of different generations and derived in a varying manner, and that they show this by their appearance, which is characteristic in every case.

If Pl. 31, figs. 10, 11, 17, 19, 20, and Pl. 32, fig. 24, be inspected it will be seen that very remarkable differences exist between the cells here drawn; all these figures are to the same scale and the cells were all found in the germinal epithelium. The same remark about differences applies to Pl. 30, fig. 6, Pl. 32. fig. 21, and Pl. 33, fig. 30. These are in a characteristic stage of the prophases of the heterotypic division, and not only do figs. 6 and 21 differ markedly in their nuclei, but the cytoplasmic inclusions are quite distinctly unlike in each example.

In the same way, if the cell divisions drawn in Pl. 32, fig. 22, Pl. 32, fig. 28, and Pl. 33, figs. 34 and 39 be compared it will be noticed that the cytoplasmic inclusions behave differently and are different in size and shape. If the spermatocyte in Pl. 32, fig. 25, be compared with that drawn in Pl. 33, fig. 32, and the spermatid in Pl. 32, fig. 24 A, with that in Pl. 33, fig. 36, it will be seen that remarkable differences exist. This brief recital of some of the curious facts which are to be found in the ovotestis of Helix at once serve to show that the problem of the derivation of the various sex cells from the indifferent cell is a very difficult matter to understand, and one which has been inadequately treated.

I should hasten to make it quite clear that this paper does not by any means completely describe all the sorts of cells found in the ovotestis. For myself, I consider that the many months of close attention which I have devoted to this work have only served to show me that a complete explanation of all the various cell generations in the snail's gonad is not the work of months, but of years. It will need the collection of a complete series of sections of gonads for every month of the year except the hibernatory ones.[1] I have found that properly to describe the remarkable facts concerning the origin, function, and fate of the nurse or yolk cells alone would need a separate paper, and should circumstances allow I hope to apply myself to this task.[2] Bolls Lee (**9**) was struck by the number and varying sizes of the spermatogonia in the snail. I can at present think of at least several sorts of spermatogonia; by this I mean that it is quite possible to find a large number of cells which are in the spermatogonial generation of the male cells, and which differ markedly either in their nucleus, their Nebenkern, or their mitochondria.

Cell Generations in the Snail.

The most important result of this study has been the realisation that in observing the mixed mass of cells in the lumen of the ovotestis, one deals not with one generation of male cells derived in the same way, but with several generations whose origins are in certain ways considerably different. It is not intended at this juncture to attempt any explanation of this until the various cells have been described as well as possible. From the excellent work of Bolls Lee (**9**) and Ancel (**2**), not to mention some of the older writers, we are fairly well acquainted with the appearance of the typical lumen of the ovotestis. Inspection of the text-figures will serve to show what these authors have failed to emphasise sufficiently, viz. that the appearance of the various alveoli differs greatly, not only individually, but just as importantly in the contents of the alveoli which are different at different levels.

In Pl. 32, figs. 28 and 29, and Pl. 33, figs. 30, 31, 32, 33, 34, 35, 36, and 37 I have drawn typical stages of the metamorphosis of a sperm from the loose cells lying in the open lower region of the alveolus.

The spermatogonial division is drawn in Pl. 32, fig. 28. Typically one gets small mitochondria often so small as to be difficult to detect, and sometimes two other bodies of a larger nature can be found. One, marked *X.N.K.*, may be the Nebenkern, but it is too small to be easily made out; the other is a large granule (*S.G.*), the history and fate of which has been so ably described by Bolls Lee (**9**). Suffice to say, this body is quite definitely seen in Flemming-fixed material stained in iron alum hæmatoxylin, and persists for a long time in the sperm cycle. The chromosomes in these divisions are seed-like, slightly elongate, and much crowded. There are considerably more than forty, and the correct number seems to be forty-eight. As far as I can tell, the mitochondria in this form of spermatogonial division do not alter in shape during kinesis. In Pl. 32, fig. 29, I have drawn a spermatogonium just after division has finished and when the nucleus is properly reformed. At *S.B.* is a spindle bridge, at *S.G.* a siderophilous granule, towards one side of the nucleus the small mitochondria are grouped into a conical heap, and finally floating free in the cytoplasm is an apparently serrate elongated body so plain and large as to be easily drawn in with the camera lucida (*N.K.*). I feel quite sure that this is the Nebenkern. When the spireme appears and the loops become grouped to form the contraction figure the Nebenkern takes up its position where the centrosome is known to be in other cases. It should be stated that in the best preparations I have, one is unable to see a centrosome at any stage until near the maturation divisions. In Pl. 33, fig. 30, the Nebenkern (*N.K.*) appears to be broken into pieces but still has the elongate rectangular shape. The mitochondria are now becoming more loosely disposed, and by the stage drawn in Pl. 33, fig. 31, are larger and dispersed throughout the cytoplasm. The Nebenkern is now quite distinctly formed of a number of tiny intensely-staining rodlets.

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[1] This has now been done.

[2] This paper (Part iv) has lately been finished.

By the end of the growth period the Nebenkern (Pl. 33, fig. 32) is seen to consist of elongate, slightly curved rodlets, somewhat irregularly disposed, but often placed end to end, as shown in the spermatid in Pl. 33, fig. 35.

These rods are most easily described as banana-shaped.

They lie in, or are grouped so as to enclose, an archoplasmic region near the nucleus.

Despite Bolls Lee's assertion, I must confess that I am quite unable to find a centrosome inside this archoplasm, but there is little doubt that such a body may be embedded in this mass.[1]

When growth stage has finished the cell may be the size drawn in Pl. 33, fig. 33, at 4250 diameters. The first maturation prophases are in progress. The chromosomes are appearing, while the Nebenkern, as such, has disappeared. In favourable examples it is found that small rodlets are still visible here and there (*N.K.* in Pl. 33, fig. 33), and these are almost certainly parts of the scattered Nebenkern. The individual rods appear to break up into minor rodlets.

Bolls Lee (**9**) described in the maturation division a remarkable centrosome structure. I found this quite easily in some of my preparations, and these triradiate bodies are seen at *A.S.* The striæ figured by Lee in these bodies I did not find so marked, but as our technique was different this is not a matter of great importance.

The mitochondria are very dense and numerous. The spindle now forms, with the disappearance of the astral body (*A.S.*), and one gets a figure in which the mitochondria are heaped around the spindle and elongated in shape as if affected by some lines of force (see Pl. 33, fig. 34).

Murray (**8**) figures the Nebenkern fragments as grouped around the poles of the astral figure, but I am unable to come to a definite opinion on this point. I am able to say that occasionally one finds little rectangular bodies which might be the Nebenkern, but unfortunately, as the mitochondria also become elongate, it is difficult to come to a decision as to the nature of these elements. Since mitochondrial stains also tinge the Nebenkern, staining tests have so far failed. The second maturation division closely resembles the first in so far as the behaviour of the plasmatic bodies are concerned. The spermatid then appears as drawn in Pl. 33, fig. 35, when it is just beginning to metamorphose. At what is later seen to be the front end of the cell, the nucleus is found to be covered at one side by a densely staining cap—the acroblast. Despite especial work in this connection, I have been unable to follow this body back into the spermatocyte. No staining method of which I know will discriminate between acroblast and mitochondrium, and it is obviously impossible to identify this body until it has taken up its position next to the front edge of the nuclear membrane. The spermatid,[1] besides containing the Nebenkern and the mitochondria, is seen to be provided with a cloud of granules of a smaller size lying behind the nucleus, near the locality from which the axial filament presently begins to grow. The granules ($M.^2$) are hardly demonstrable till the spermatid is in the stage drawn in Pl. 33, fig. 35, but from thence onwards they are quite easily found.

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[1] Subsequent work shows that Lee is correct.

As the spermatid lengthens, the nucleus becomes shaped as shown in Pl. 34 , fig. 41, the acroblast lying as a thickened area laterally. In Pl. 33, fig. 36, I have carefully drawn a spermatid at this stage. The nucleus has become blackly stained with iron hæmatoxylin, while the small granules, which will be called micromitochondria, are closely grouped behind the nucleus. The other mitochondria, which, to distinguish them, will be called the macromitochondria, lie further back. At the letter $C.^2$ is a structure, constantly present, formed of a large and a slightly smaller granule. The larger granule is probably the second centrosome; the other might be a mitochondrial granule, but I cannot advance any definite evidence as to its nature. A stage just before this is drawn in Pl. 34, fig. 42, and the nucleus does not yet stain entirely basophil. In Pl. 34, fig. 43, a still later stage is drawn. The micromitochondria ($M.^2$) are now grouped around the axial filament a good part of its way, and the centrosomic structure is still visible.

In Text-fig. 4 iii a later stage is drawn; in this preparation the two bead-like bodies in the tail of the sperm were particularly obvious.

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[1] See Addendum A.

In Pl. 34, fig. 44, at a still later stage, the Nebenkern, which hitherto kept its rectangular figure, has become collapsed by the pressure of the narrowing space in which it lies, and its individual parts are better revealed. In some cases, at least, it seems that the Nebenkern elements at this stage do really enclose an archoplasmic region, or at least a denser region of the cytoplasm. In Pl. 34, fig. 44, the mitochondrial elements are becoming larger, and they now form a rough coat to the axial filament. First are seen the micromitochondria, which now form an undoubted covering, while behind is generally seen the Nebenkern. Behind this lie the macromitochondria, which are less evenly disposed than the micromitochondria. By Pl. 34, fig. 45, the micromitochondria have disappeared as such, but if the axial filament is carefully examined, it will be seen that it has increased both in thickness or bore, and in its affinity for basic dyes. Followed further down to the macromitochondrial region the filament gradually resumes its original staining powers and proportionate size. These areas of the filament are marked *X*, upper, *Y*, where the intermediate region lies, and *Z*, lower, where there is a slight but hardly perceptible thickening. In Pl. 33, fig. 37, at a higher power is drawn the front region of a metamorphosing sperm just before the stage described in Pl. 34, fig. 44. The disposition of the elements is quite typical, while the Nebenkern is crumpled up and is seen to consist of nine batonettes or rods, which together formed the rectangular structure drawn in the previous figure (Pl. 33, fig. 36). In Pl. 33, fig. 37, these rods seemed to be surrounded by a clear zone.

As metamorphosis goes on the mixed-up macromitochondria and batonettes become further and further removed from the head of the sperm, while the former of the two become somewhat larger and increasingly fewer in number. They seem to be absorbed finally into the sheath of the tail, and if anything is cast off it must be a very small portion indeed.[1]

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[1] A residuual bead is always cast off, and it always contains some mitochondrial grains.

As the mitochondria slough down the sperm tail, the mitochondrial sheath becomes thicker and more darkly staining in the region just cleared of the chondriosomes, the natural inference being that the latter form the sheath. This seems supported by such a stage as that drawn in Pl. 34, fig. 45, at *X*, *Y*, and *Z*.

In the areas where the cells of the male generation are densely packed one often finds variations in Nebenkern, mitochondria, and nucleus. Without at present going into the question of the cause of these variations, whether technical or inherent in the snail, it is proposed to describe them. In Pl. 32, fig. 25, is drawn a spermatocyte near the end of growth period. The mitochondria are seen to be small, hollow spheres, scattered throughout the cytoplasm in much the same way as in the spermatocyte in Pl. 33, fig. 32.

The Nebenkern elements are much more numerous than in the cases already described, where there are generally thirty rodlets in the spermatocyte, and from six to twelve in the spermatid. In the other spermatocytes, such as that in Pl. 32, fig. 25, the rodlets are always almost completely circular, that slight curve or banana shape being here much exaggerated. The circular rodlets generally are placed as shown in Pl. 32, fig. 25, but it will be noticed that they are almost invariably placed so that their outer, thicker edge lies outermost, and their centre is in contact with the archoplasm. This is very well seen in Pl. 32, fig. 24 A. Re-examination of the Nebenkern elements in Pl. 33, figs. 32, 35, and 36 shows that in this variety of cell the elements are banana-shaped, and the convex surface or back of the rodlet is turned inwards—exactly the opposite of what is found in Pl. 32, figs. 25 or 24 A, etc. Now when the spermatocyte breaks into the prophases, the cell looks like Pl. 32, fig. 27. The chromosomes are appearing ; the Nebenkern elements have become disposed into two groups, one on each side of the nucleus, evidently being influenced by the centrosome. Below the nucleus lie several other rods, curved so as to form a circle, but with one side especially thickened. It is quite characteristic of these stages that at this period one has difficulty in distinguishing the mitochondria, which are sometimes quite big and ring-shaped, from the Nebenkern elements.

As will be seen on inspection of Pl. 32, fig. 24 A, the mitochondria may be quite large and appear as hollow spheres. When the cell division is in the metaphase the mitochondria and Nebenkern elements become mixed, and I am unable to say that the asters in any way affect the rodlets, as has been claimed by Murray.[1] In some cases one seems to be satisfied that the spindle-fibres do exert some influence; in others the opposite seems to be the case. In almost, if not all, metaphases I have examined, I feel with regard to this question that the Scotch verdict, "not proven," is the safest view to take. Should a specific stain either for mitochondria or for the rodlets of the Nebenkern be found, it may be possible to throw some clearer light upon this question.

In Pl. 32, fig. 22, a second maturation division is shown. The mitochondria are hollow spheres, appearing as ringlets, and here and there lie the elements of the Nebenkern, which I can positively identify. The spermatid of this small-rodlet generation almost always has a nucleus which contains a greater number of karyosomes[2] than the sort drawn in Pl. 33, fig. 35. The Nebenkern elements in the example drawn in Pl. 32, fig. 26, were very small, and at this stage evidently just collecting after cell division. It will be noted in all these stages except the one drawn in Pl. 32, fig. 27, that the cell is angular in shape, from the abutting neighbouring cells pressing on each side.

When the spermatid begins to metamorphose the micro-mitochondria appear, and the Nebenkern is formed as shown in Pl. 32, fig. 24 A. Curiously enough, the sperm head in the early stages of its transformation is generally irregular in shape—probably traceable to the cramped quarters in which the cell is forced to metamorphose.

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[1] I have since found that in division the batonettes do lie in the zone of the asters.

[2] See Pl. 34, fig. 48 *a*, *b*, *c*, where spermatid nuclei from different regions are drawn to show variation in karyosomes. The bulk of the karyosome matter does not vary much, only number.

In Text-fig. 1, iii, iv, the two extremes in these various kinds of cells are drawn. Fig. iii shows the typical Nebenkern formed of many curved rods, while fig. iv shows the typical rectangular Nebenkern formed of fewer elements.

The remarkable differences which are found in the later spermatids are illustrated in Pl. 33, figs. 37 and 40. These are not quite at the same stage, the latter being slightly younger. In this form the Nebenkern is very large and peculiar, differing most markedly from that in Pl. 33, fig. 37. This also applies to the mitochondria, which, while being larger in Pl. 33, fig. 40, and fewer. But, as will be noticed in Pl. 32, fig. 26, largeness of mitochondria and smallness of Nebenkern batonettes do not necessarily go hand in hand.

The Manner of Metamorphosis of an Indifferent Germinal Epithelial Cell into a Primary Spermatogonium.

Many of the older writers thought that the main factor of change in the epithelial nucleus was the appearance, or at least the exaggeration, of the chromatin lumps. Pl. 31, figs. 11 and 19 show this. In one case we deal with a spermatogonium (the latter), and in the other with a oogonium. But it should at once be pointed out that, from a careful study of these stages, I have concluded that the amount of real

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Fig. i.—Part of the lumen less well supplied with nutriment. The epithelial cells have here and there grown into progerminative cells (*X.*), passed to the prophases of the heterotypic division (*C.F.*) and afterwards have begun to drop off (*X.Y.*). (Text-fig. 4.) At *X.* and *E.N.C.* are yolk cells in stages of disintegration. At *N.C.N.* is a bare yolk cell nucleus. At *S.N.* are many spermatogonial nuclei lying in a syncytium (*R.*), a quite characteristic occurrence in these localities of a lumen. As at *S.* and in other cells there is a distinct cloud near the nucleus. (See Pl. 31, figs. 19, 20; Pl. 32, figs. 21 and 23.) Fig. ii.—Passage of a male progerminative cell (primary spermatogonium) from its place on the germinal epithelium

TEXT-FIG. 3.

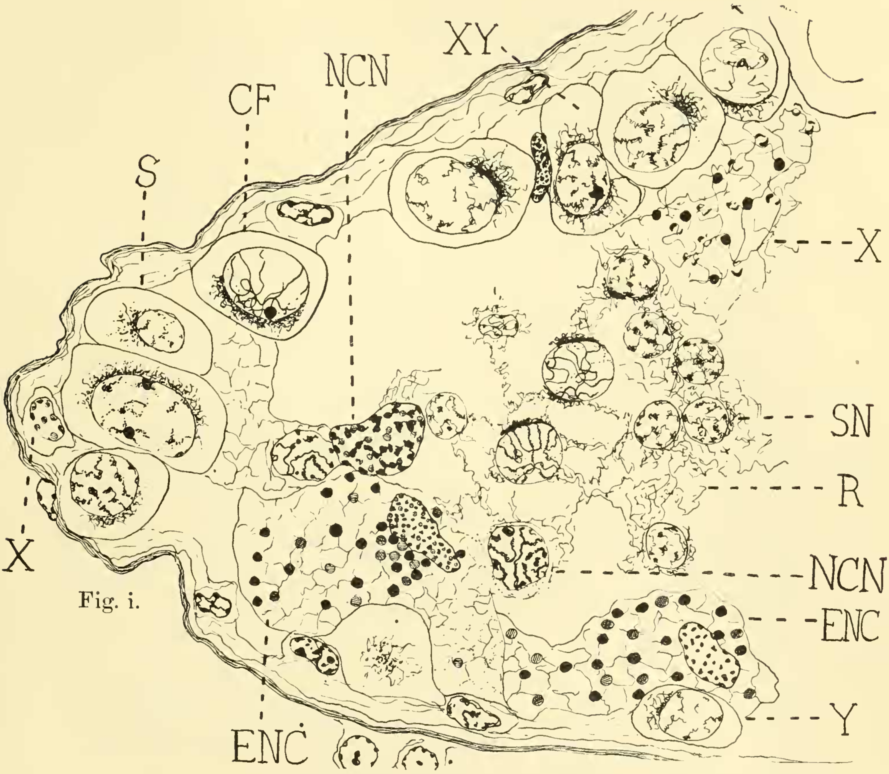

Fig. i.

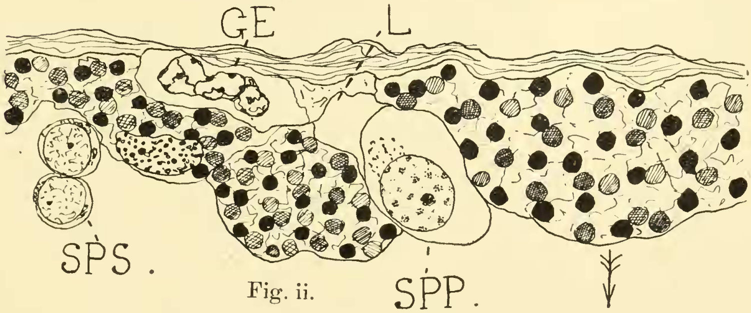

Fig. ii.

(*L.*) into the lumen of the diverticulum. At *S.P.S.* are secondary spermatogonia derived from a division of such a cell as *S.P.P.* The arrow points to the inside of the lumen.

variation is quite remarkable, and no two epithelial cells metamorphose in quite the same way.

In Pl. 30, fig. 2, the upper nucleus has to its right a small, darker area in the cytoplasm, and the same applies to the upper part of the middle nucleus in this figure. Not all cells possess this cloud—or, more correctly, I should say that I have been unable to find the cloud in every epithelial cell. The cytoplasm of the germinal epithelial cell is of the open variety, and is best seen in preparations stained in iron alum-alizarin-toluidin blue, where it is a purplish-blue in shade.

In some cases, when the cell is about to abandon its indifference, the nucleus, at first a depressed oval, becomes spherical or semi-spherical, as shown in Pl. 31, figs. 17 and 19. I believe that of the many variations which one finds, all fall roughly under three heads: In the first, one has a cell in the positions marked *X.* in Text-fig. 1, i, growing into fig. ii, *X.*, in the same text-figure. Also in other cases one finds such an example as that of Text-fig. 2 at 1 and 2. While, again, one always finds cases such as that drawn in Text-fig. 3, i, *X.Y.*, and Text-fig. 4, i, and in Pl. 29, fig. 1, at the roman figures (i, ii, iii).

In the other cases one finds the progerminative cell as in Text-fig. 3, ii, *S.P.P.* Also it seems that these arbitrary classes are linked up by other classes, such as the cell 3 *b* in Pl. 29, fig. 1 (explained in "Discussion").

In every case of an epithelial cell of the snail metamorphosing into a germ-cell of either sex one finds two constant facts: one is the appearance of a fine cloud in the cytoplasm, which follows after the other—an enlargement of the nucleus. It is only by fine fixing reagents that this cloud is shown, but it has never before been described simply because the workers on the snail destroyed it with acetic acid or alcohol.

I am unable to say why this cloud, which is shown in Pl. 31, figs. 11 and 19 in a very early stage, should with absolute constancy be found to one side of the nucleus. It may be that it is on the side near which lies the centrosome if that be present, but I am unable to advance any other opinion beyond this: I believe the cloud is formed by a growth and enlargement of the zone already indicated as being present in the cytoplasm of at least some germinal epithelial cells (Pl. 30, fig. 2, *Y.* and *X.*), which is quite possibly a conglomeration of some material around or in an attraction sphere. But so difficult is it to study these early stages that I cannot advance any evidence based on actual knowledge of this small aggregation in the cytoplasm.

One significant fact I can, however, point out: it is that the growth or the appearance of this faint cloud is subsequent upon a change in the size and often of the staining power of the nucleus. The latter moves first; the cloud then appears.

In every case the germinal nucleus at first becomes round or oval, and the chromatin lumps, before connected here and there by bridges, become spherical and isolated. After this the cloud in the cytoplasm becomes marked. From this stage onwards there is a difference in the behaviour of the kinds of male cells derived from such progerminative cells. In the case of one generation of male cells, shown in Text-fig. 3, the chromatin lumps, after some slight changes, break into a spireme, and the prophases of the heterotypic division are undergone while the cell still adheres to the germinal epithelium (*C.F.* in Text-fig. 3, i). The same sort of occurrence invariably happens in the oocyte, where a spireme gradually appears and the prophases take place in situ. But in the case of certain cells shown in Text-fig. 2, ii, and 3, ii, the behaviour of the chromatin is different. It can at once be explained that this different behaviour is due to the fact that such cells are going to undergo mitosis. For this to happen the chromatin must come into a resting stage for the formation of a reticulum, which soon breaks up into chromosomes. (See Text-fig. 2, ii, at 5.)

In this last cell the chromosomes are beginning to appear. It has been customary for many writers on this subject to describe minutely changes in the nucleus which herald either the formation of a spermatogonium or an oogonium, whichever the case may be. I do not fail to recognise the splendid labours of such observers when I state that I cannot accept anything in their descriptions of such changes. There may be very slight differences, but I have failed to find any upon which one could reliably base a dogmatic statement. The differences between the individual behaviour of the chromatin in the nuclei of a number of progerminatives of the same probable future sex, are so wide as to cover the statements depending on size, staining power, and arrangement of chromatin lumps, upon which these descriptions are based.

To return to the cells which I have mentioned as about to undergo mitosis, Text-fig. 3, ii, *S.P.P.*, shows a section through a region from which male cells were appearing when the snail was killed. The cell *S.P.P.* is leaving its place in the epithelium marked *L* and is pushing out. This cell has a nucleus, oval in shape and containing its chromatin in faintly staining lumps. There is a cloud in the cytoplasm containing dark bodies (Pl. 34, fig. 49). Inspection of Text-fig. 2 at the figure 2 shows another cell, but in a different locality, free

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TEXT-FIG. 4.

Fig. i.—Shows dropping off of the male cells, about to finish growth stage. At *Y.* the cell is becoming detached, at *X.* it is already in the lumen. The cell *Z.* may belong to this generation, or from the generation to which the boqnet stages (*BS*) are derived. There is generally a mixture of various generations in this lower region of the diverticulum. × 900. Fig. ii.—Bichromate smear of adult sperm head. *A.* = acrosome, *N.* = nucleus, *C.* = centrosome, *M.* = mitochondrial sheath. Iron hæmatoxylin. × 2000. Fig. iii.—Group of metamorphosing spermatids showing definite position of Nebenkern (*N.K.*). Also the curious arrangement of two granules (*C.*) where the hind centrosome lies. (Compare Pl. 34, fig. 44.) × 1000. Fig. iv.—A spermatogonial group with Nebenkerne (*N.K.*), spindle bridge (*S.B.*), mitochondria (*M.*), and a yolk cell (*NC.*). (Compare Pl. 29, figs. 1, 2, and Pl. 32, fig. 29.) Fig. v.—Several batonettes greatly enlarged showing difference in shape and size, and in one the relations of the archoplasm (*A.R.*) with the inside of the batonette. (See Pl. 32, figs. 24 A and 25.) Fig. vi.—The large variety of Nebenkern rod at same magnification. (See Pl. 33, fig. 36.) Fig. vii.—Three spermatogonia attached to a cell with a germinal epithelial nucleus, but with the cell granules grouped together at *X.* What these are, whether Nebenkern batonettes or mitochondria, was not ascertained. × 2000.

Text-fig. 4.

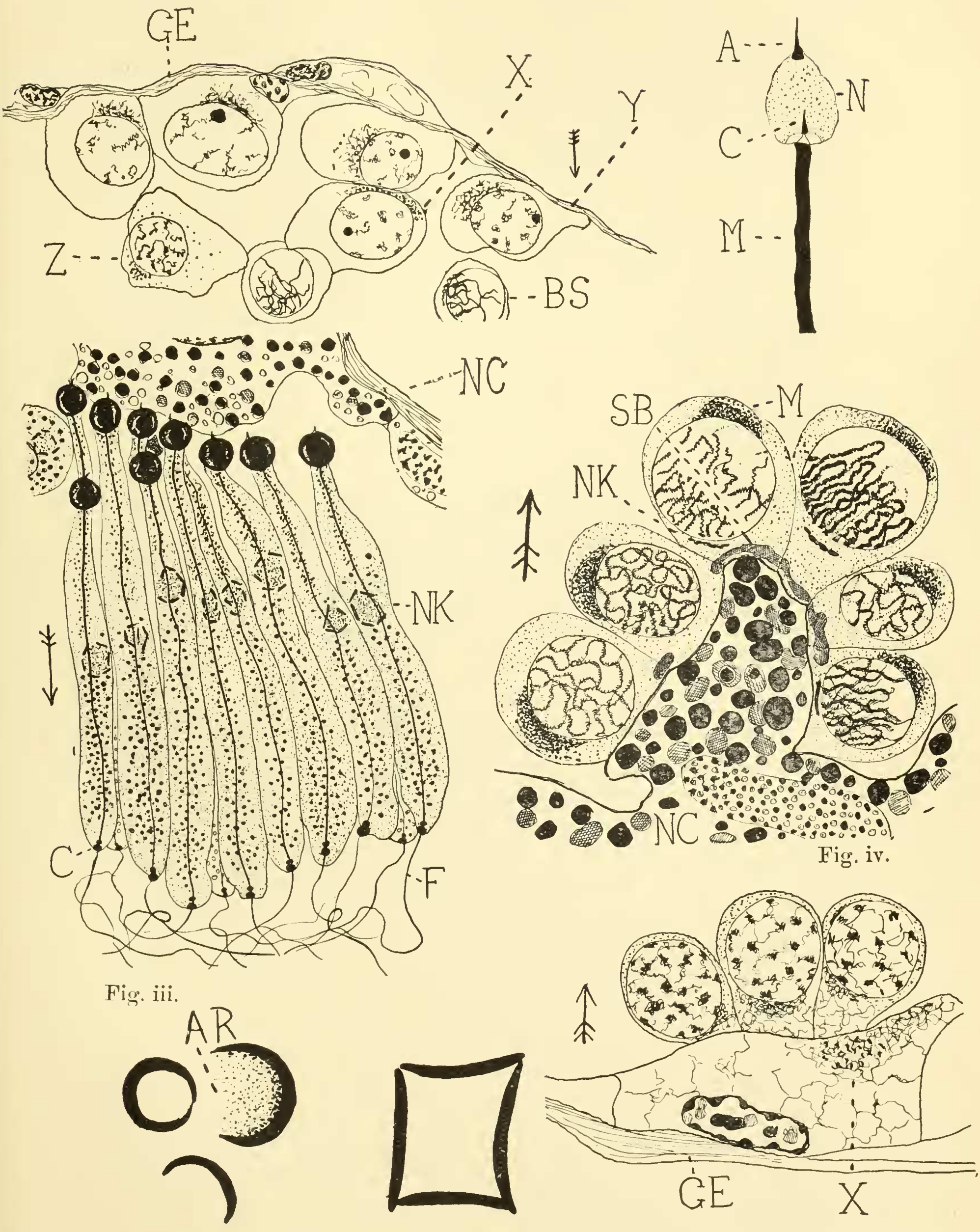

Fig. i.

Fig. ii.

Fig. iii.

Fig. iv.

Fig. v.

Fig. vi.

Fig. vii.

from much yolk. This cell is drawn in Pl. 31, fig. 17, at a very high power.

The nucleus has the same general appearance as in Text-fig. 3 at *S.P.P.*, though in the latter the chromatin lumps have not broken up so much as yet, as in Pl. 31, fig. 17. Both these cells will ultimately divide and give rise to some secondary cells. The answer to the question why there should be differences in these two cells, however small, is that they originate from parts of the germinal epithelium which are in a different nutritive condition. Cells like these soon divide many times and give rise to bunches of spermatogonia such as those shown in Pl. 29, fig. 1, at the outer 2, or in Text-fig. 4 at iv. The connection between the yolk cell and the male progerminative cell is sometimes severed, sometimes retained.

Now, if attention be centred on the cytoplasmic cloud in these progerminative cells it will be noted that apart from the apparent denseness of the cytoplasm which causes the cloudy appearance one finds distinct granules. These are apparently the first signs of the mitochondria. The size and number of these granules are very variable. In Pl. 31, fig. 17, they were very large and distinct.

Towards the time when the lumps of chromatin in the nucleus have fragmented to form a fine clear granular structure these cytoplasmic bodies become dispersed more and more from the zone from which they originally appeared.

The chromosomes soon appear and a cell division takes place, the cytoplasmic bodies being visible and scattered here and there around the amphiaster. As far as I have been able to ascertain, these mitochondria do not lose their rounded shape during division. Such a series of divisions finally give rise to cells such as those drawn in Text-fig. 3 at *S.P.S.*

After a division is finished, and these secondary cells have regained their resting nucleus an examination of the cytoplasm generally shows that two sets of bodies are present: one, the mitochondria, are scattered indiscriminately, the other is a dark structure lying close to the nuclear membrane. In some generations of secondary spermatogonia this latter structure is not demonstrable till later. This body is the first visible sign of the Nebenkern of Pulmonates, and it may possibly have been present in the stage drawn in Pl. 31, fig. 17, but may not be plain enough to make its detection possible.

In Pl. 32, fig. 24, is drawn a spermatogonium of the generation shown in Text-fig. 2, fig. ii, at 2. The Nebenkern (*N.K.*) is quite easily seen to consist of several straight rods. In Pl. 32, fig. 29, the Nebenkern of another generation of spermatogonia is shown. The fate of the Nebenkern and mitochondria of these generations has already been followed out.

Another sort of male generation of cells appears in the following way: In Text-fig. 3, i, is drawn a lumen in which nearly all the germinal epithelial cells have thrown off their indifference and have become large peculiar cells. The epithelium from which such cells arise consists of a single layer of cells, yolk cells being either exhausted or completely absent. This is shown in the plan on Pl. 29, fig. 1, at the Roman figures i–iv.

In Pl. 31, fig. 19, the cell at ii in Pl. 29, fig. 1, is drawn at high magnification. It lies on the epithelium projecting into the lumen in a different way from Pl. 31, fig. 17.

The nucleus still has the chromatin lumps which stain somewhat lightly, and there are two nucleoli which are hollow spheres, as far as one can ascertain. At any rate, their centre does not stain so heavily as the periphery. The lumps gradually become elongated and join up to form a wide reticulum (Pl. 31, fig. 20). Now, the plasma in fig. 19 is seen to contain to one side of the nucleus, the usual zone which here and there has a granule. In the next stage the granules have become quite plain and are seen to consist of the ring-like mitochondria already described from the figures in Pl. 31. In fig. 20 the zone of thickened cytoplasm has spread right around to the opposite side of the nucleus, but as yet no distinct mitochondria have appeared on this side (*Z.*).

There is rarely any other structure to be made out in the cytoplasm, but in a few cases one finds what seems to be an archoplasmic mass (*A.R.*) upon which may be stuck from two to four Nebenkern rods. This is so seldom found at this stage that I do not think it is the rule. The wide open reticulum now breaks into a loose spireme and a contraction figure is shown in Pl. 32, fig. 21.[1] In this cell the mitochondria were rather irregular in shape, here and there undoubtedly ring-like, but they were all collected to one side of the nucleus towards where the chromatin filaments converged. It will be noticed that the nucleoplasm in this kind of male cell generation is abnormally large for the amount of chromatin spireme. The diplotene stage and the rest of the prophases soon take place and the growth period begins. The Text-fig. 3, i, shows that at *X.Y.* and at other parts these cells are losing their attachment to the walls. In Text-fig. 4, i, the cell *Y.* is just falling into the lumen, while *X.* has already arrived there. The contraction figures below belong to another cell generation. In Pl. 32, fig. 23, a typical cell just after the beginning of the growth stage is shown. The attachment to the germinal epithelium consists only of a small area (X_1) which will soon part. The mitochondria are dispersed in the cytoplasm, and outside the nuclear membrane (at *N.K.*) is a cloud in which can be seen embedded a very large number of small Nebenkern rods. These are slightly curved. This is the usual way in which the Nebenkern appears in this generation. Pl. 32, fig. 25, is a later stage. Subsequent stages are drawn in Pl. 32, figs. 22, 25, 26, and 27, and have been described.

It seems that in this generation the nucleus after the prophases collapses somewhat in size, proportionate to the normal amount of chromatin contained therein. It will be noted that no spermatogonial divisions take place.

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[1] Pachynema.

The Manner of Metamorphosis of an Indifferent Germinal Epithelial Cell into an Oogonium.

It has been agreed almost unanimously among workers on the ovotestis that the presence of a group of yolk cells is the determining factor in the appearance of the female cell. I consider this explanation inadequate, for it can be shown that spermatogonia appear in regions choked with yolk cells, and oogonia may appear in regions where little or no yolk is present. I feel that it would be quite a mistake to entertain the view that a yolk cell exclusively determines the awakening of an indifferent cell into the egg generation. But it might be true to say that the presence of abundant yolk was the sine quâ non for the successful growth of an oocyte to maturity. It would also be quite true to say that in the majority of cases the transition from an indifferent cell to an egg took place behind or between some yolk cells. Despite careful observation of a large number of cases I find it difficult to formulate a statement of any differences between male and female nuclei till after the growth stage begins. This is not the case with the cytoplasm. The drawing in Pl. 30, fig. 6, was thought by me to be an oogonium in contraction stage, firstly because it was so closely embedded behind yolk cells that its exit into the lumen would have been difficult, and secondly because the epithelium in this region was seen to be producing many oocytes.

What I take to be the Nebenkern (*N.K.*) is very like that in the male cell drawn at a little later stage in Pl. 33, fig. 31, while the mitochondria are not very characteristic. The nucleus is unlike the male generation nucleus drawn in Pl. 32, fig. 21, but almost identical with that drawn in Pl. 33, fig. 30. Apropos of the statement that the spermatogonia appear in yolk regions compare Text-fig. 1, x, and especially Text-fig. 3, ii, at *S.P.P.* On the other hand, in an epithelium like that in Text-fig. 2, ii, a solitary oocyte may grow. This all shows that the matter is somewhat more complicated than at first supposed. Having shown that to the end of the prophases the nucleus does not give us any very characteristic evidence we will turn to the cytoplasmic bodies. In Pl. 31, fig. 11, is drawn a cell thought to be an oogonium. I believe it to be such for the same reasons which I brought forward for fig. 6 of Pl. 30. The cytoplasmic cloud does not differ markedly from many another example known to be a male—it contains the same lumps and is isolated to one side of the nucleus in the same way. Pl. 30, fig. 6, is thought to be a later stage. A Nebenkern has appeared. Fig. 10 of Pl. 31 is a still later stage—the nucleus is losing its chromatin loops, while the mitochondrial mass looks less granular and more flocculent. At one side is an undoubted Nebenkern, but of a slightly different type, the rods being straighter. Fig. 10 of Pl. 31 really corresponds to the same stage in the male drawn Pl. 33, fig. 31.

I have already mentioned what great variation was found in the appearance of the male cells. This, I think, applies even more strongly to the case of the female cells, but a difference which seems to exist between all the female cells and the male cell is that in the latter the mitochondria are from the first to the last granular and comparatively large, while in the former the mitochondria, even if at first granular, rapidly become flocculent and lie like a cloud, as shown in Pl. 31, figs. 10 and 12, and in later stages in Pl. 30, figs. 3, 5, 7, Pl. 31, figs. 9, 13, 14. Later, the mitochondria of the egg seem to become granular, spherical, and often somewhat larger than the male, but there is then no possibility about making a mistake as to the sex at this period.

Pl. 31, figs. 12 and 9, show stages in the mitochondria. In the former figure, which is drawn one-half the size of fig. 10, the mitochondria are dispersing, not, however, from a centre as they are in fig. 9. In this figure there is a centre from which flocculent masses of mitochondria radiate. It will now be clear that the most certainly diagnostic evidence for difference between the oogonium and spermatogonium is to be found in the mitochondria, which early behave differently in either sex.

The Fate of the Mitochondria and Nebenkern in the Egg.[1]

The flocculent mitochondria gradually become dispersed somewhat unevenly throughout the cytoplasm as shown in Pl. 30, figs. 3 and 7, and in Pl. 31, fig. 9. If these mitochondria be examined it will be found that they consist of masses of exceedingly fine grains (Pl. 31, fig. 13). In later stages these grains become here and there mixed up with larger granules as shown in Pl. 31, fig. 14. In a still later stage no flocculent masses remain, there being now only large mitochondria. The latter seem to be derived from the flocculent masses as shown in Pl. 31, fig. 14, and in different eggs are of different sizes as is the case with the mitochondria of the male. Soon after this, vacuoles appear in the cytoplasm and the mitochondria lie in the trabeculæ between these alveoli (Pl. 31, fig. 16). Yolk disclets sooner orlater partially fill these spaces (Pl. 31, fig. 16, *Y.*), but are not to be confused with the mitochondria at any stage. In later stages the cytoplasm becomes divided into a cortical layer without yolk vacuoles, and an inner layer like that drawn in Pl. 31, fig. 16.

After the stage drawn in Pl. 31, fig. 10, the Nebenkern seems generally to become obliterated by a curtain of mitochondria, but as shown in Pl. 30, fig. 5, it may still be quite plain. Its subsequent fate is difficult to follow, for it cannot be found in every oocyte. In Pl. 31, fig. 22 (at *X.N.K.*) are round bodies which are almost certainly separate parts of the Nebenkern. I have found many oocytes showing these ring-like structures.

In later stages there appears a clearer zone near one side of the nucleus, and in this zone appear several blocks of darkly-staining matter as shown in Pl. 31, fig. 18; the two left-hand pieces are drawn at a high power in Pl. 30, fig. 8, and consist of more or less solid matter, which generally contains cavities. The nature of these structures and their connection, if any, with the mitochondria or Nebenkern, is unknown to me.

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[1] See Addendum B.

The Differentiation of an Indifferent Germinal Epithelial Cell into a Nurse or Yolk Cell.

It is quite usual to find small yolk granules in any indifferent epithelial cell, and when the cell becomes either an oogonium or a spermatogonium, these soon disappear at first (see Pl. 30, fig. 6). The growth of an indifferent cell into a yolk cell is accompanied by the appearance of abundant yolk disclets, and a change in the size and in the disposition of the chromatin of the nucleus. In the latter the chromatin bridges between the angular lumps if present (Pl. 30, fig. 2) become lost and the chromatin becomes formed into little round structures. Synchronously with the appearance of more and more yolk, the nucleus grows larger and larger till it may be 35 μ in length.

These changes are easily noticed and have already been described well (**2**). A more difficult problem is what happens to the plasmatic bodies of the modified cell. Does a Nebenkern appear? What happens to the mitochondria? It is not at all easy to find answers to these questions. In Pl. 33, fig. 38, is drawn a cell with a yolk cell nucleus gathered into numerous chromatin lumps. In the cytoplasm there were no yolk disclets, but the reticulum was of the very wide kind, peculiar to yolk cells. Here and there were small masses containing mitochondria (*M.*), and to the left of the nucleus was what I took to be an attraction sphere, inside which were embedded a number of dark bodies, probably the Nebenkern. In many ways this cell is intermediate between the yolk cell and the indifferent epithelial cell. In the full yolk cell it is probably not possible to discover attraction sphere, Nebenkern, or mitochondria. But after many of the yolk discs have been absorbed by neighbouring cells, it is generally possible to discover bodies shaped like those drawn in the oocyte in Pl. 31, fig. 12, at *X.N.K.* What these are, subsequent work will be needed to show, but I have done enough to think that modified representatives of Nebenkern and mitochondria may be found in the cytoplasm of the yolk cell.[1]

After some time the yolk cells become exhausted completely, and the wide reticulum breaks up and the nuclei float out into the lumen of the ovotestis diverticulum as shown in Text-fig. 3, i, *N.C.N.*, *E.N.C.* These nuclei do not degenerate, as might be supposed. They lie there in the midst of a mass of live cells and degenerate yolk, and seem to undergo further changes which need not detain us at present, but I should say that it is my firm opinion that these nuclei regain a cytoplasm and become spermatocytes.

Description of the Scheme on Pl. 29, fig. 1.

In this figure I have united my final conclusions concerning the processes which go on in the various regions of diverticulum of the ovotestis of Helix. All cells have been drawn in to scale with a camera lucida, and in the majority of cases are the same as those on the other plates drawn at a higher power. Great care has been taken to show the germinal epithelium in its true state; thus, for instance, the region of the right lower edge marked by the Roman figures is the same kind as that drawn in Text-fig. 3, i. The left lower half appears also in Text-fig. 2, i, and so on.

In the following description, after mentioning each series of cells, I will indicate where they are drawn at a higher power in the other plates. If not the identical cell, I will show this by adding the letter *W.* to the bracket.

The Genesis of the Egg.

A = Differentiating germinal cell embedded in yolk cells (Pl. 31, fig. 11–*W.*).
B = Young oocyte (Pl. 30, fig. 6–*W*).
C = Older oocyte (Pl. 30, fig. 3, 7, etc.–*W.*).
D = Older oocyte (Pl. 31, fig. 9–*W.*).

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[1] More work on a dozen species of Pulmonates shows that the true yolk cells do not contain mitochondria or Nebenkern.

The Genesis of the Sperm.

In the Roman figures to the right I have indicated the sort of male cell generation in which no spermatogonial divisions take place. (See also Text-fig. 5.)

I. Earlier stage than drawn in any other figures.
II. Progerminative cell (Pl. 31, fig. 19).
III. Mitochondria appearing in spermatogonium. Rare kind of Nebenkern (Pl. 31, fig. 20).
IV. Spermatocyte, with Nebenkern just appearing (Pl. 32, fig. 23). Usual Nebenkern.
V. End of growth stage. Cytoplasm differs greatly in size in different examples (Pl. 32, fig. 25–*W.*).
VI. Maturation division. Spindle curiously orientated with relation to cell, first maturation. (Second maturation drawn in Pl. 32. fig. 22–*W.*).
VII. Spermatid (Pl. 32, fig. 26).
VIII. Later spermatid (Pl. 32, fig. 24 A–*W.*).

It will be noted that on the left bottom region at the Arabic numbers 1 and 2, there is another source of male cells. The two sources of cells, marked in Arabic and in Roman numerals respectively, are often mixed indiscriminately and later stages are hard to distinguish. Thus the bouquet stages marked 3 *a* are certainly derived from such a source as 1, 2, on the left, because in the generation derived directly as shown at the Roman numerals the contraction figure and other stages up to the beginning of growth take place directly on the wall (see Pl. 32, fig. 21, which was stuck on the wall, and Text-fig. 3, i).

In the case of the cells marked V 4 it was not possible to tell whether they were derived as in the Roman numerals or as in the Arabic. Thus the Roman numerals beyond VI are probably of uncertain derivation. VI itself, on account of its great size, is probably of the Roman numeral generation. Almost invariably the two generations, derived respectively from the bottom left corner at 1 and 2, and from the bottom right at the Roman numerals have a Nebenkern formed of numerous curved elements as in Pl. 32, fig. 25. Moreover, the size of the entire spermatocyte, though not of its nucleus, varies greatly (VI, V, V–4). The spermatocytes are either crowded as in V–4 to VIII or loose as at V. Crowded spermatocytes have walls like that in Pl. 32, fig. 25, etc., loose ones like that in Pl. 32, fig. 27. Nebenkern in each may be the same. The spermatid may be a large vacuolated cell, though the sperm has the same end result.

At the Arabic numerals are drawn all the various generations which have male progerminatives which divide to give rise to secondary spermatogonia and even tertiary generations of spermatogonia.

At the mid left-hand side at 1 is a primary spermatogonium dropping into the lumen. The male progerminative cell at II on the right at the Roman numerals is derived from a different kind of epithelium with a semi-empty lumen. At 2 (left side) this primary spermatogonium gives rise to many secondary cells. Some, as at 2 *a*, go on dividing till they become very small. Others form the bunch drawn at the outer middle 2 (Text-fig. 4, iv).

By 3 the prophases of the heterotypic division have begun. One sometimes finds cells like that drawn at 3 *b*. Isolated cells like this are found in connection with a nurse-cell and may form a link between the generations at 1 in Arabic numerals and between the other in Roman. These cells, such as that at 3 *b*, seem to be primary spermatogonia which have failed to divide but have entered the prophases.

At 4 are the spermatocytes; in this generation they never vary so greatly as in the generation derived from the epithelium, as at (I, II, III) or as in the crowded cells. At 5 is a first maturation division, which can be compared with the first maturation at VI.[1] The spindles are the same size.

The following indicates where these cells are drawn at a higher power:

1 in Pl. 34, fig. 49 (*W.*).
2 in Pl. 32, fig. 29.

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[1] Such cells are not all so large as this example.

2 *b* in Pl. 32, fig. 24 (*W.*).
2 *c* in Pl. 32, fig. 28 (*W.*).
3 in Pl. 33, fig. 31 (*W.*).
4 in Pl. 33, fig. 32 (*W.*).
5 in Pl. 33, fig. 34 (*W.*).
6 in Pl. 33, fig. 35 (*W.*).
7 in Pl. 33, fig. 36 (*W.*).
8 in Pl. 34, fig. 45 (*W.*).
9 in Pl. 34, fig. 46 (*W.*).

Discussion.

(*a*) Nebenkern.—Sex determiner, spindle former, degeneration product, chromidium, are only a few of the different characters supposed to be fulfilled by this curious aggregation of stick-like structures, known as the Nebenkern.

In the first place we will examine the evidence that the Nebenkern determines the sex of a differentiating cell. Demoll, tracing the Nebenkern from about the time of the "Bukett stadium" in the prophases of the heterotype divisions, thought that the subsequent development of this body was the lever which turned the cell to oogonium. It is quite true that it is at or immediately after the bouquet stage that the first definite evidence for the female sex[1] can be found, but had Demoll succeeded in following out the mitochondria he would have seen that the latter may show a differentiation towards one sex before the Nebenkern becomes in any way characteristic. Demoll's reservation that the "sex chromosome" probably influences the Nebenkern to act in a way productive of one sex or the other is interesting, but supported by no evidence.

Briefly stated, I should think the following disposed of the "Nebenkern and sex" hypothesis[1]:

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[1] Sex, female or male, I have used somewhat loosely instead of "metamorphosis of indifferent cell into oogonium or spermatogonium" respectively.

(1) The mitochondria in the egg are often diagnostic before the Nebenkern rods alter.

(2) The Nebenkern varies more in individual spermatogonia than between Demoll's spermatocyte and oocyte examples.

(3) The whole train of events leading to the appearance of undoubted Nebenkern and of mitochondria is so liable to variation that it would be impracticable to look to such structures as sex determiners.

(4) The Nebenkern may be late in appearance, even after the cell is undoubtedly male.

(5) Bouquet stages are not rightly to be considered as definite milestones, parallel in the sex development of either sperm or egg cells, for the bouquet stage may appear in the male at a time when it is known that the sex[1] has been determined cell generations ago. I refer in this to the secondary spermatogonia which go on dividing and which sporadically enter growth stage.

(6) The amount of Nebenkern material in the bouquet stages of the male alone varies from "none demonstrable" to a large amount.

It will be seen, therefore, that lack of proper examination and of sufficient knowledge of the Nebenkern has led Demoll to suggest a theory unsupported by evidence of any description.

What would certainly be more logical would be to say that the alteration in the Nebenkern was the result of and not the reason for the appearance of a definite sex. As far as diagnosis goes the mitochondria are better objects for building up theories, but for many reasons the last paragraph about the Nebenkern also applies to the mitochondria. As for the suggestion that the sex-chromosome guides the Nebenkern in its "choice of sex" I have no evidence either for or against. Demoll's idea that up to the appearance of the Nebenkern the cell may be considered indifferent is disproven by every species of evidence.

The only other likely suggestion as to the function of the Nebenkern is one which has been freely supported by certain observers. It is that these rods are the condensed spindle, and that their supposed disappearance before mitosis is due to the fact that they have gone to form the astral figure.

As far as the snail is concerned I am somewhat doubtful as to the validity of this interesting suggestion. Without definitely condemning or upholding this view I think that the following facts should be borne in mind. In the Helicids :

(1) It is, if not absolutely unproven, at least extremely doubtful whether the rods do disappear[1] (Pl. 32, figs. 23 and 27).

(2) The substance of the rods differs in bulk, as also do the number of the rods. Variation in the size of the spindle is almost negligible (Figs. 24 A and 36, Figs. 25 and 32, etc.).

(3) In the prophases the rods can undoubtedly be found lying here and there in disorder, not as if they served a definite function in the formation of the amphiaster.

(4) In many spermatogonial late telophases the Nebenkern appears in a position removed from the centrosome, archoplasm, or spindle bridge. Not in any case as if it appeared to be reinstated by a condensation of any part of the amphiaster (Pl. 32, fig. 29).

(5) In numerous other animals the spindle appears and disappears without a Nebenkern (batonettes).

(6) Bolls Lee and I describe a triradiate structure from which the astral rays arise, and which is undoubtedly unrelated to the Nebenkern (Pl. 33, fig. 33).

(7) Spermatogonial generations are to be found in which the cells before entering mitosis had no definite Nebenkern.

Before the spindle-forming function of the Nebenkern can be proven all these objections must be met. I do not think any of them can be explained satisfactorily from the point of view of the observers who espouse the theory. Terni's statement already mentioned, that he did not believe that all the idiozomatic material was taken up in the formation of the spindle, I think rather vitiates the evidence for the view that it is necessarily the Nebenkern part of the idiozome (archoplasm) which goes to form the middle spindle. Terni's material seemed to admit of clearer study of the Nebenkern than mine, because in the snail the mitochondria do not appear to become localised to one side of the cell as often happens in Geotriton, according to Terni. This localisation often clears the area around the Nebenkern, and assists observation. (For further evidence against the "Nebenkern spindle" view see Fauré-Frémiet, page 580 and fig. 33).

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[1] In a forthcoming paper I have described how to fix and stain so as to show these rods during metaphase.

To return to the Nebenkern of Helix aspersa, it may be worth while to attempt to analyse some of the variations so strikingly evident.

The Nebenkern rods differ in number without a doubt; they likewise differ in size, and their shape is seldom the same. Neither the number nor the great difference in size can be the result of varying conditions of fixation. For the shape I cannot speak with such confidence. It is quite possible that some small variation either in the fixative or the condition of the cell might produce distortion of the rods. To test this I examined a great deal of material by the intra-vitam methods. Janus green of the strength of 1 in 30,000 and neutral red about the same strength were used. These did not give very good results. After many experiments I found that intra-vitam methods would not properly settle the question, because the rods of the Nebenkern never seemed to take the stain heavily enough.

I devised the following "fresh method": A small part of the ovotestis was smeared on a slide, and a little 1 per cent. permanganate of potash was added. A coverslip was then placed over this, and the preparation, after about sixty seconds, showed the cell inclusions a brown colour, and they were very easily studied. The mitochondria were remarkably clear, and were of the spherical type, not stick or rod-like. The Nebenkern was found to vary as in my drawings—rod-shaped or curved. In this permanganate method the cells are killed instantly. I found the oocytes stained just as shown in my diagrams.

The Probable Function of the Nebenkern.

It will be seen that for some reasons I am at present unable to accept the view that the Nebenkern has anything to do with the formation of the astral figure. Terni's important paper, I feel, reinforces me in this (see especially his figures 15 to 18).

I do not find myself in a position better than that of many of my predecessors in-so-far as an analysis of the function of the Nebenkern is concerned. But I feel sure that its real rôle has not been pointed out. It seems evident that it is, in molluscs, a piece of cell mechanism as definite as the mitochondria. I have shown that, like the mitochondria, it is liable to variation in time of appearance, in size, and in its general behaviour. It generally stains like the mitochondria, it is destroyed by the fixatives which also destroy mitochondria, and it is seen best in preparations which show the latter best. I therefore think that the Nebenkern rods in Helix are to be classed with the two sorts of mitochondria described by me as definite plasmatic elements whose exact function is still unknown, but which have nothing to do with the amphiaster. The aster and centrosomes can be followed out best in material unfavourable for a study of the mitochondria or Nebenkern.

With regard to staining reactions, Fauré-Fremiet says: "Si l'on isole des spermatocytes dans le sérum au chlorure de manganese additionné de violet dahlia ou de violet de gentiane, les mitochondries se colorent assez rapidement en lilias, tandis que le Nebenkern reste quelquefois plus longtemps avant de se colorer."

b. Mitochondria.

It is not intended to discuss these bodies at length, but it may be pointed out that in the snail one finds a remarkable phenomenon. It is the presence of two definite kinds of mitochondria, which apparently have a definite location in every sperm tail. This is a fact of cardinal importance, and seems to show that there is a division of labour or function between the mitochondria of the spermatid. The position of the micromitochondria is more definite than that of any plasmatic structure other than centrosomes or perforatorium.

Terni figures and describes his mitochondria throughout as rods, remarkably equal in size and length. He has observed fresh material, and therefore apparently this rod-like structure, which can be artificially produced in Helix by bad fixation, is the true state of affairs in Geotriton.[1]

c. The Determination of Oogonium, Spermatogonium, or Nurse-Cell.

In the following, the words "determination of sex" are used for the above for convenience. I have been unable to produce any definite evidence regarding the determination of sex. This paper deals almost exclusively with the plasma, but I have come to certain conclusions with regard to this important matter. In short, I am convinced that since it is the nucleus which is the first part of the indifferent cell to begin differentiation, we are justified in believing that the nucleus is the prime factor in the production of any one sex. The nucleus enlarges, its chromatin undergoes changes, a cloud appears at the edge of the nucleus, evidently under the influence of the latter, and every step in the metamorphosis of the cytoplasm is preceded by one in the nucleus. If we saw the nucleus remaining as it was for some time, waiting till the Nebenkern or mitochondria appeared, we would naturally think that the latter structures, after appearing, stimulated the nucleus. As it is the other way about, I feel justified in entertaining the view that it is not the plasma, but the nucleus, which induces differentiation along a special path. The natural outcome of this suggestion is the word "sex-chromosome." Curiously enough, after examining many hundreds of sections I have never seen mitosis in the germinal epithelium. When I consider that I only found very few cases of divisions of the primary spermatogonia, I am reluctant to make a statement which might be injudicious, but I cannot overlook the fact that there appears to be abundant evidence that the germinal cells divide amitotically, and no evidence for mitosis. It was my desire to examine the cytoplasm of a germinal epithelial cell in mitosis, if it could be found, for at this stage many bodies become revealed.

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[1] Since this paper was written I have come to the conclusion that the rod-like mitochondria (Pl. 33, fig. 34) where they occur are artefracts produced by bad fixation, but as I have not observed Triton I cannot venture to question Terni's results.

Any evidence that one might have for believing that all nuclei in the germinal epithelium of the ovotestis are endowed with the same potentialities—that is, for maleness, femaleness, or for becoming a nurse-cell—and that this power is directed in any one of the three channels by purely external or environmental conditions, is somewhat hypothetical. The favourite view, that abundant yolk cells (nutriment) affects the decision, seems negatived by the fact that male progerminative cells can, and do, appear in areas choked with yolk. A number of cells stuck upon the germinal epithelium seemed too big to be male cells, and their cytoplasm recalled that of the spermatocyte. I was much puzzled by these, and it has occurred to me that they might be intermediates between spermatocytes and oocytes. They contained distinct Nebenkern batonettes of the semi-lunar type, and mitochondria of a fine nature, but not flocculent. The cells were much larger than a full-grown spermatocyte, and were located in a yolkless area of the epithelium. Could it be possible that the indifferent cell is affected by stimuli sent forth by the presence of yolk cells, by crowded spermatogonia, and by the general condition of that area in which it lies? It seems certain that the matter is complicated, and cannot be reduced to the bald statement that oocytes appear because of abundant nutrition.

I think that there are a variety of conditions which act on the cell; the latter is, so to speak, hanging in the balance when it begins to differentiate. If the stimulus is not definite enough an intermediate might be formed, and later degenerate, as many cells do. For the moment I can think of several possible sources of stimulation. Abundant and differentiating male cells in the lumen might send the balance towards femaleness. Absence of any cells in the lumen might stimulate towards maleness. As a matter of fact, here often enters a direct contradiction of the real facts. I have several cases where a lumen, quite or almost empty, is producing either male or female cells alone. It should be said, however, that in the latter case the lumen wall still had some few yolk cells.

Finally, I believe that the nucleus of the indifferent cell may be stimulated by a variety of external agencies to tend towards one sex, and that the nucleus is the cell organ responsible for the differentiation of the plasmatic elements. I conclude that the plasmatic elements do not influence the nucleus in this matter.

d. The Different Sperm Generations in the Ovotestis.

The ovotestis lumen is continually giving rise to new sperm cells. The conditions of nutriment alter so much that very few of these generations arise under the same stimuli.

The number of times which the primary spermatogonium divides, and the number of times the secondary cells continue fission, depends on the nutrimental conditions of the lumen. I think that the reasons for variation of the generations are as follows:

(1) Spermatogonial divisions of variable number, leading to a variation in Nebenkern and mitochondria. It is a fact that such variation does arise through differing numbers of divisions.

(2) The conditions of nutriment profoundly affect the appearance of the plasmatic structures in spermatogonia or

male progerminatives, and are probably instrumental in checking or stimulating spermatogonial divisions.

(3) The nutrimental conditions of the wall producing a male generation is hardly ever the same.

(4) Finally, it will be seen that the spermatogonium beginning growth period may have had a very variable history. This is demonstrated by the variation in not only its plasma, but often in its nucleus.

It can safely be said that in hardly any other group are the conditions under which the male cells arise so open to variation.

Difference in the mitochondria of the various generations is principally due to the fact that a much divided line of spermatogonia contains but few mitochondrial granules. When growth begins, in every case it seems that the mitochondria increase in number by dividing; but there is some difficulty in observing this, though one knows this process must happen by the fact that spermatogonia have fewer mitochondria than spermatocytes, and the question is not merely a matter of increase of size of the granules in the growth period. Thus the spermatogonium, which has, so to speak, originated at the tail end of a large number of divisions, starts out on growth with fewer mitochondria. These divide, but never give rise to the same number of granules as one finds in other "less divided" generations; but paucity in numbers is almost always balanced by increased size of individual granules (compare Pl. 33, figs. 37 and 40).

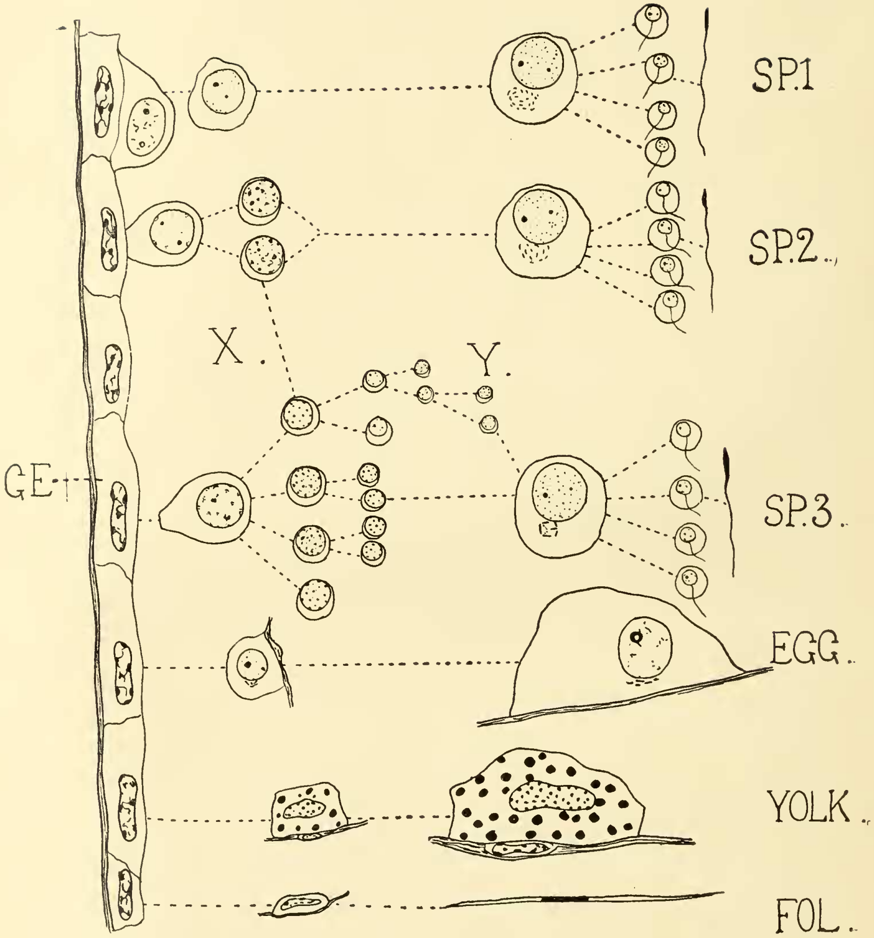
In many of the drawings in this paper mitochondria are shown as hollow spheres, while in others they look quite solid (compare Pl. 32, figs. 25 and 26). The most natural explanation of this is, that for some reason the stain is more easily extracted out of some granules than out of others, and that all the mitochondria are really hollow spheres, though overstains tinge the medullary zone. To test this I took a slide with "solid" mitochondria and extracted more of the stain, examining it at intervals. The half of the slide which was over-differentiated showed most of the mitochondria as hollow spheres. But not everywhere. Those parts where the mitochondria still seemed solid were generally seen to have their cell elements slightly distorted or run together. I conclude that there is good evidence for believing that the mitochondria of Helix consist of an inner, somewhat chromophobe substance, and a cortical stainable area.

In Text-fig. 5 is drawn a diagrammatic scheme of the derivation of the various cell elements in the ovotestis from the indifferent epithelium. At *S.P.* 1 is the generation in which no spermatogonial divisions occur, the cell dropping into the lumen just when the growth stage has been entered. At *S.P.* 2 is a progerminative cell, which only divides once. Its elements may go on to the growth stage, or may (as at *X.*) divide again several times. At *S.P.* 3 the much-divided generation of spermatogonia is shown. At *Y.* the spermatogonia are still dividing, and have become very small. According as to the stage when the spermatogonial cells enter the growth period their cytoplasmic bodies have various more or less evident differences. The connecting link between *S.P.* 1 and *S.P.* 3 is provided by such a cell as that in *S.P.* 2, whose divisions may be curtailed. The diagram is based on evidence deduced from observation of the spermatogonia, but there is no manner of finding out how many times such a spermatogonium as that at *S.P.* 3, *Y*, has divided.

Below *S.P.* 3 are drawn the derivations of egg, yolk cell, and follicle cell from the epithelium. The latter is not proportionate in size to any of the cell elements in the figure.

It has been shown that the number and size of the Nebenkern batonettes vary greatly. In some cases, such as the generation marked in Pl. 29, fig. 1, by Roman numerals, the large number is never reduced by spermatogonial divisions, since these do not occur. The spermatocyte, therefore, has the original large number which appeared in the progerminative cell. It seems certain that the individual batonettes do not increase in number before a spermatogonial division, so that after such a division their number is halved approximately.

TEXT-FIG. 5.



Scheme showing generations of spermatogonia (*S.P.* 1, 2, and 3). At *X.* generation 2 and 3 are interconnecting, at *Y.* are the much divided, small spermatogonia. The four small cells to the right of the spermatogonial generations are spermatids. Below are the derivations of egg, yolk cell, and follicle cell from the germinal epithelium (*G.E.*).

In the spermatogonia derived after many divisions the batonettes ultimately form the rectangular figure drawn on Pl. 34. They are fewer in number, but, as often happens with the mitochondria, generally bigger, though not invariably (compare Pl. 32, fig. 24 A, and Pl. 33, fig. 36).

Curiously enough, in different examples the archoplasm seems to be variably demonstrable, and these differences do not depend altogether on the depth of penetration of fixative, and the consequent possibility of different degrees of staining.

Murray (**8**) considers that the archoplasmic and batonette material are interconnecting, the rod being, as it were, a thickened edge of the idiozome. This is apparently true in some cases (see Text-fig. 4, *V.A.R.*). In others the batonette is separate. I do not think that this exceptional apparent intercommunication between idiozome and Nebenkern rods is evidence in favour of the view that the batonettes are a part of the spindle apparatus.

SUMMARY.

(1) The ovotestis of Helix aspersa is formed of finger-like diverticula. The latter are hollow at their lower ends, which connect to the hermaphrodite duct, while the upper ends contain more yolk, and are filled completely with metamorphosing male cells.

(2) According to the manner of derivation—that is, the nutrimental conditions of the locality from which new cells arise, and the number (if any) of times which these new cells divide—there are quite wide differences in the individual generations derived from and under such varying conditions.

(3) These differences are found in nucleus, mitochondria, Nebenkern, and general cell volume.

(4) The mitochondria vary in size and number, and such variation seems to be caused by the varying number of spermatogonial divisions in different regions of the ovotestis.

(5) In the early spermatid smaller mitochondria, the micro-mitochondria appear in an unknown way near the region from which the axial filament takes its origin from the centro-

some applied to the nucleus. These micromitochondria are about one-fourth the size of the other, or macromitochondria. No perceptible variation in size of the micromitochondria of various generations has been found.

(6) The micromitochondria form the front sheath of the sperm; the hind region of the micromitochondrial sheath intercommunicates with the macromitochondrial sheath, which follows behind.

(7) The Nebenkern does not apparently become absorbed into the substance of the mitochondrial sheath. A sloughing off appears to take place.

(8) The minute cytology of the derivation of the sperms, eggs, and nurse-cells is described.

(9) The determination of the sex of the indifferent cell seems to be brought about by a variety of causes. The explanation of femaleness by presence of yolk cells is held to be inadequate, for male progerminative cells also appear in regions choked with yolk.

(10) The probable function of the Nebenkern is discussed.

Addendum A.

With regard to the body in the spermatids (Pl. 32, fig. 24 A, and Pl. 33, fig. 35) marked *P.N.A.* it has lately been found that this structure is derived from a number of grains, which in the case of Arion I have traced back to the young spermatocyte. In Helix aspersa these granules could not be found in the spermatocyte. *P.N.A.* stands for post-nuclear apparatus, from the position of this structure. The latter is fully considered in a forthcoming paper.

Addendum B.

With regard to the cytoplasmic bodies in the egg, some late papers by Schaxel ('Zool. Jahrb.,' Bd. xxxiv, etc.) are of interest. Schaxel claims that the nucleus emits chromatin into the cytoplasm at a brief period after the prophases of the heterotypic division. He gives several stages (primary chromasie, chromasie post emission, etc.) during which the egg is being formed. He finds no cytoplasmic bodies till after the bouquet stage, an obvious error of observation, and an error committed by Miss Beckwith ('Journ. Morph.,' xxv) as well. His figures of emission are (in Aricia) merely late stages in formation of the mitochondria (compare Pl. 30, fig. 3 of this paper and Schaxel's Pl. 16, figs. 9, 10, etc., in his paper 'Zool. Jahrb ,' Bd. xxxiv). He overlooks the early stages of mitochondria formation, while his description of the "extra-nuclear chromatin" is apparently in some of his papers merely a misinterpretation of later stages of the formation of the mitochondria.

Miss Beckwith, in a paper quoted above, throws doubt on the "Chromatin-emission" of Schaxel. Miss Beckwith says: "There is no evidence of formed material passing through the nuclear membrane into the cytoplasm either early (Schaxel) or late (Smallwood) in the growth period." It will be seen that Schaxel's work needs confirmation. To me this observer's papers appear to be written in a partisan manner, simply to bolster up the "chromidia hypothesis" in its application to the Metazoa. For an excellent review of this matter see Dobell, 'Quart. Journ. Micr. Sci.,' vol. 53.

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9. Lee, Bolls.—“La Cellule,” ts. xi, xiii, xx, xxi, various papers on the cytology of Helix.

EXPLANATION OF PLATES 29–34,[1]

Illustrating Mr. Bronté Gatenby's paper on “The Cytoplasmic Inclusions of the Germ-Cells”: Part II, Helix aspersa.

Explanation of Lettering.

A. Acrosome (perforatorium). *A.R.* Archoplasm (idiozome). *A.L.N.* Nucleus of Ancel's layer (mesoderm). *A.S.* Astral sphere (from which each aster arises). *C.* Centrosome. *C.W.* Cell wall. *F.N.* Follicle nucleus. *G.E.* Germinal epithelium. *M.* Ordinary mitochondria (macromitochondria). M^2. Smaller mitochondria (micromitochondria). *N.C.* Nurse- (yolk) cells. *N.K.* Nebenkern (rods or batonettes). *S.B.* Spindle bridge. *S.G.* Siderophilous granule. *S.P.G.* Spermatogonium. *V.A.C.* Alveoli in substance of cytoplasm of egg. Later become filled with yolk discs. *X.N.K.* Body thought to be Nebenkern rod. *Y.* Yolk disclets. (Only in certain figures, for others see text.)

All figures drawn with camera lucida, paper at table level. Koritska $\frac{1}{15}$th semi-apochromatic oil immersion and compensating eye-pieces were almost invariably used. In most cases the figures are reduced. The arrow points to the inside of the lumen.

F.W.A. Fixation in Flemming without acetic acid. *C.* Fixation in Champy's fluid. *S.* Bichromate osmic smear.

PLATE 29.

Fig. 1.—For an explanation see the text page 36. × 800.

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[1] In such a figure as that in Pl. 32, fig. 24 A, the mitochondria seen at a lower focal level have been drawn in palely, as is a usual cytological convention. In Pl. 33, fig. 33, the white line in each mitochondrial grain represents the “light.” Hollow mitochondria are as drawn in Pl. 33, fig. 40.

PLATE 30.

Fig. 2.—Germinal epithelium showing a cell of the mesoderm (Ancel's layer, *A.L.N.*) and three germinal nuclei. At *X.* and *Y.* are what are probably attraction spheres. × 4250, *F.W.A.*

Fig. 3.—Oocyte lying in germinal epithelium, mitochondria beginning to disperse (*M.*). × 2000, *F.W.A.*

Fig. 4.—Part of germinal epithelium showing yolk cells (*N.C.*), a germinal nucleus (*G.E.*), and a spermatogonium (*S.P.G.*). × 2000, *F.W.A.*

Fig. 5.—Upper part of an oocyte showing the Nebenkern (*N.K.*) and the dark mass of mitochondria just before dispersal in cytoplasm (*M.*). × 2000, *F.W.A.*

Fig. 6.—Bouquet stage of a supposed oocyte. This cell was deeply embedded in yolk like that in Pl. 29, fig. 1 A or B. × 4250, *F.W.A.*

Fig. 7.—Another oocyte showing mitochondria (*M.*) dispersing and yolk being deposited (*Y.*). × 2000, *F.W.A.*

Fig. 8.—Represents the two left-hand bodies marked *X.* in Pl. 31, fig. 18. × 4250, *C.*

PLATE 31.

Fig. 9.—Oocyte showing dispersal of mitochondria from a centre (*c*) near the nucleus. × 650, *C.*

Fig. 10.—Oocyte just after the stage drawn in Pl. 30, fig. 6. × 4250, *F.W.A.*

Fig. 11.—Supposed oogonium embedded in yolk cells like that in Pl. 29, fig. 1 A or B. × 4250, *F.W.A.*

Fig. 12.—Oocyte at time of dispersal of flocculent mitochondria, showing several bodies (*X.N.K.*) supposed to be Nebenkern elements × 2000, *F.W.A.*

Fig. 13, 14, 15, and 16, several stages in the development of the cytoplasm of the egg. The upper white area in each figure represents the nucleus. Fig. 13 was drawn from the same egg as that in fig. 9; this was about 100 μ in length. Egg in fig. 14 was 140 μ. Fig. 15 about 150 μ. Fig. 16 about 120 μ. × 2000, *F.W.A.* and Champy.

Fig. 17.—Male progerminative cell showing development of mitochondria. × 4250, *C.*

Fig. 18.—Oocyte near end of growth stage, to show the location of the bodies drawn in Pl. 30, fig. 8, at a higher power. × 510, *C.*

Fig. 19.—Male progerminative cell showing appearance of cloud and granules. × 4250, *C.*

Fig. 20.—Later stage. Archoplasm (*A.R.*) and Nebenkern rodlets of fairly rare type. Usual sort is shown in Pl. 32, fig. 23, and appears later. *Z.* Zone of cytoplasmic activity which has spread around the nucleus. × 4250, *C.*

PLATE 32.

Fig. 21.—Bouquet stage of generation drawn in the two preceding figures. *M.* Mitochondria. × 4250, *C.*

Fig. 22.—Second maturation division showing probable Nebenkern rods (*X.N.K.*) in cytoplasm mixed with mitochondria. × 4250, *C.*

Fig. 23.—Later stage, showing appearance of Nebenkern (*N.K.*) and dispersal of mitochondria; at *X.* the cell is losing its place on the germinal epithelium. × 4250, *C.* (Compare Text-fig. 4, i.)

Fig. 24.—Secondary spermatogonium showing Nebenkern (*N.K.*) and mitochondria. × 4250, *F.W.A.*

Fig. 24 A.—Spermatid with ring-like Nebenkern (*N.K.*) and large mitochondria. × 4250, *F.W.A.*

Fig. 25.—Spermatocyte near end of growth showing Nebenkern with many curved rods. × 4250, *C.*

Fig. 26.—Spermatid with largish mitochondria and small curved batonettes in Nebenkern. Nucleus has large number of nucleoli. × 4250, *C.* (For *P.N.A.* see Addendum A.)

Fig. 27.—Spermatocyte in prophases. Ring-like Nebenkern rods scattered somewhat haphazardly. At *X.*, *X.*, region of centrosomes. × 4250, *F.W.A.*

Fig. 28.—Spermatogonial division with seed-like chromosomes. *X.N.K.* Probable Nebenkern. × 4250, *F.W.A.*

Fig. 29.—Spermatogonium (secondary) with Nebenkern (*N.K.*). × 4250, *F.W.A.*

PLATE 33.

(All *F.W.A.* × 4250.)

Fig. 30.—Bouquet stage, showing loops radiating towards Nebenkern. Rather small example.

Fig. 31.—Growth stage of spermatocyte. Mitochondria dispersing. Nebenkern at *NK.*

Fig. 32.—Near end of growth period. Nebenkern rods banana-shaped. Between thirty and forty in number.

Fig. 33.—Prophase showing astral body (*A.S.*), which will give rise to part of the spindle. Mitochondria at this stage often rod-like. Nebenkern has lost its original disposition.

Fig. 34.—First maturation division metaphase, showing shape of mitochondria and general difficulty of detecting Nebenkern rods.

Fig. 35.—Spermatid with small mitochondria and straight or slightly curved Nebenkern rods. Nucleoli few in number. (For *P.N.A.* see Addendum A.)

Fig. 36.—Spermatid of same generation.

Fig. 37.—Later spermatid showing collapse of Nebenken structure.

Fig. 38.—Probable nurse-cell showing nucleus and cytoplasmic bodies. No yolk disclets remain (or have been formed). The exact history of this cell is difficult to make out (see p. 591).

Fig. 39.—Spermatogonial division with few large mitochondria. Compare Pl. 32, fig. 28.

Fig. 40.—Spermatid with ring-like Nebenkern rods and large mitochondria.

PLATE 34.

Figs. 41, 42, 43, 44, 45, and 46. × 2000.

Figs. 40 to 45 stages in the formation of the sperm.

Fig. 46.—Sperm from smear drawn to scale of the foregoing figures (*S.*).

Fig. 47.—Spermatid × 2000, showing manner in which axial filament grows in cramped quarters. The micromitochondria keep their definite position.

Fig. 48.—Spermatid nuclei × 2000, showing variations found in chromatin nucleoli. These hold good without much variation for every nucleus in the bunch of spermatids.

Fig. 49.—Male progerminative cell (primary spermatogonium) of the generation drawn in Text-fig. 3, ii, *S.P.P.* × 4250.